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(54) Title: METHOD OF MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY OR PHYSIOLOGY USING CDC25 SUBSTRATES

(57) Abstract

The present invention provides a method for accelerating and increasing the production of biomass, branches, flowers and fruits, and for modifying one or more plant morphological, biochemical and physiological properties or characteristics, such as one or more environmental adaptive responses and/or developmental processes of plants, by ectopically expressing therein a Cdc25 substrate or modified Cdc25 substrate, in particular a Cdc2 protein or a homologue, analogue or derivative thereof.

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METHOD OF MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY OR PHYSIOLOGY USING CDC25 SUBSTRATES

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying plant morphological, biochemical and physiological properties or characteristics, such as one or more environmental adaptive responses and/or developmental processes, said method comprising introducing into a plant cell, tissue or organ a gene capable of expressing a cell cycle control protein, in particular a cyclin-dependent kinase (CDK), such as, for example a substrate of Cdc25 or a modified substrate of Cdc25, and, in particular Cdc2a, or a homologue, analogue or derivative thereof, and generating a whole plant therefrom. The present invention extends to gene constructs which are useful for performing the inventive method and to transgenic plants produced therewith having altered morphological and/or biochemical and/or physiological properties compared to their otherwise isogenic counterparts.

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

BACKGROUND TO THE INVENTION

In light of the dwindling supply of arable land available for agriculture, intensive crop production is an imperative for the purposes of feeding the increasing worldwide population. To achieve this end, it is necessary to increase the rate of plant development and/or the total biomass and vigour of crop plants.

There are plethora of data showing that the external application of plant hormones has profound effects on development, metabolism and environmental fitness. For example, the external application of cytokinins produces a variety of morphological, biochemical and physiological effects in plants, including the stimulation of organogenesis, shoot initiation from callus cultures, release of lateral buds from apical dominance, dwarf growth, alteration of source/sink relationships, stimulation of pigment synthesis, inhibition of root growth, and delay of senescence. Additionally, exogenous cytokinin application following anthesis in cereals enhances grain set and yield and the phase of nuclear and cell division in the developing endosperm of cereal grains is accompanied by a peak of cytokinin concentration, suggesting a role for cytokinins in grain development in cereals (Herzog, 1980; Morgan *et al.*, 1983). Cytokinins have also been implicated in promoting the initiation of tuber formation in potato (International Patent Publication No. WO 93/07272) and in improving the resistance of potato plants to insects (United States Patent No. 5, 496, 732) and in inducing male sterility and partial female sterility in tobacco plants (European Patent No. EP-A-334, 383).

The effect of cytokinin on plant development and morphology may be attributed, at least in part, to modified biochemistry of the plant, such as a modification to the source/sink relationship in the plant or plant part.

Attempts to modify plant cytokinin-mediated and/or gibberellin-mediated growth and developmental responses employ the exogenous application of cytokinin and/or gibberellins respectively. Such approaches are costly and produce undesirable pleiotropic side-effects on the plant tissue.

Other approaches to modifying plant cytokinin-mediated growth and developmental responses employ the ectopic expression of an introduced bacterial isopentenyladenosine transferase (IPT) gene (International Patent Publication No. WO 93/07272; United States Patent No. 5, 496,732; United States Patent No. 5, 689, 042) under the control of a strong constitutive promoter sequence, developmentally-regulated promoter sequence or hormonally-inducible promoter sequence. Alternatively, plant cytokinin-mediated growth and developmental responses have been modified by the ectopic expression of the *Agrobacterium rhizogenes RolC* gene (European Patent No. EP-A- 334,383).

Previously, it had been shown that constitutive expression of yeast Cdc25 in tobacco resulted in precocious flowering, more flowers per flowering head, and the presence of "petalless" flowers alongside normal ones, in addition to pleiotropic developmental changes, including altered positioning of the leaves (International Patent Publication No. WO 92/09685; Patent WO 93/12239).

Modified plant growth or architecture has been shown to be effected by modifying expression of the G1 cyclin D3 (International Patent Publication No. WO98/42851). G1 cyclins are required for the induction DNA replication (S phase), and the induction of G1 cyclin expression is dependent on mitogenic signals such as, for example, cytokinin and sucrose (Riou-Khamlichi *et al.* 1999, Soni *et al.* 1995). As with the constitutive expression of Cdc25 in plants, prior art methods for modifying plant growth and architecture using these cyclin-encoding genes include pleiotropic effects.

The cell cycle is central to the process of growth and cell division in all multicellular organisms. The four phases of the cell cycle in eukaryotic cells (S, G2, M and G1) are

driven by a common class of heterodimeric serine/threonine protein kinases. These kinases consist of one catalytic subunit (cyclin dependent kinases or CDKs) and one activating subunit (cyclins). A large number of putative cell cycle genes have been cloned in plants over the last 7 years. A most recent count identified 17 types of cyclins and 5 types of CDKs in the model plant *Arabidopsis thaliana*. Identifying which of these genes are involved in the cell cycle is a major challenge to plant cell cycle work.

Substrates of Cdc25, including the A-type CDK, CDC2a, are homologues of the fission yeast p34 $^{Cdc2/CDC28}$ protein kinases. These polypeptides are characterised by a PSTAIRE motif in the N-terminal protein sequence, which is considered to be essential for cyclin binding (Ducommun *et al.*, 1991).

In all cells, the switch that raises activity of Cdc2a at entry into mitosis is the Cdc25 catalysed removal of phosphate from threonine-14 and/or tyrosine-15 in Cdc2a. Control of CDK activity can be achieved by cyclin association and phosphorylation. The phosphorylation of CDK can either have an inhibitory effect of an activating effect on its activity depending on the position of the phosphorylation site. The p34 ^{Cdc2} protein is regulated by an activating phosphorylation during the transition from quiescence to active cycling at threonine-167 and by inhibitory phosphorylations at tyrosine-15 and/or threonine-14 during G2 (Jacobs, 1995). In yeasts, there is only one CDK (Cdc2) and the Cdc25-catalysed removal of phosphate from threonine-14 and/or tyrosine-15 in Cdc2 occurs only once in the cell cycle, at the G2/M phase transition.

Higher eukaryotic cells contain several CDKs (animal and plant cells) and Cdc25 proteins (animal cells). Mironov *et al* (1999) and Segers *et al* (1997) provide a public listing of plant CDKs, which is incorporated herein by way of reference.

In mammals, the molecular switch of Cdc25-catalysed removal of phosphate from theonine-14 and/or tyrosine-15 in Cdc2 is also used at entry into S phase, however a separate CDK (CDK2) and a separate Cdc25 (Cdc25a) perform this function.

In plants, whilst it is known there are several CDKs it is not known if there is a single CDK that is controlled at S phase, like CDK2 by the status of threonine-14 and/or tyrosine-15 phosphorylation.

SUMMARY OF THE INVENTION

In work leading to the present invention, the present inventors sought to develop an improved method of increasing plant growth and vigour by modifying cytokinin-mediated and gibberellin-mediated plant growth and development.

Surprisingly, the inventors discovered that by introducing a genetic sequence encoding a cyclin dependent kinase (CDK) protein or a modified form thereof into plant cells, tissues or organs, in particular, a nucleotide sequence encoding a substrate of Cdc25 or a modified substrate of Cdc25, operably under the control of a plant expressible promoter sequence such as, for example, a cell-specific promoter, tissue-specific promoter, inducible promoter sequence, organ-specific promoter sequence, or a cell cycle specific gene promoter sequence, one or more cytokinin-mediated and/or gibberellin-mediated characteristics are modified in the transformed plants, without the undesirable pleiotrophy of the prior art.

The ectopic expression of a substrate of Cdc25 or a modified substrate of Cdc25, and, in particular, Cdc2a or a homologue, analogue or derivative thereof, can produce a range of desirable phenotypes in plants, such as, for example, by modifying one or more morphological, biochemical, or physiological characteristics as follows:

(i) modification of the initiation, promotion, stimulation or enhancement of cell division; (ii) modification of the initiation, promotion, stimulation or enhancement of DNA replication;(iii) modification of the initiation, promotion, stimulation or enhancement of seed set and/or size and/or development; (iv) modification of the initiation, promotion, stimulation or enhancement of tuber formation; (v) modification of the initiation, promotion, stimulation or enhancement of shoot initiation and/or development; (vi) modification of the initiation, promotion, stimulation or enhancement of root initiation and/or development; (vii) modification of the initiation, promotion, stimulation or

enhancement of lateral root initiation and/or development; (viii) modification of the initiation, promotion, stimulation or enhancement of nodule formation and/or nodule function; (ix) modification of the initiation, promotion, stimulation or enhancement of bushiness of the plant; (x) modification of the initiation, promotion, stimulation or enhancement of dwarfism in the plant; (xi) modification of the initiation, promotion, stimulation or enhancement of pigment synthesis; (xii) modification of source/sink relationships; (xii) modification of the initiation, promotion, stimulation or enhancement of senescence; and (xiv) modification of stem thickness and/or strength characteristics and/or wind-resistance of the stem.

As used herein, unless specifically stated otherwise, the term "modification of the initiation, promotion, stimulation or enhancement" in relation to a specified integer shall be taken as a clear indication that the integer is capable of being enhanced, increased, stimulated, or promoted, or alternatively, decreased, delayed, repressed, or inhibited.

Of these effects, the targeted ectopic expression of a substrate of Cdc25, in particular Cdc2a or a homologue, analogue or derivative thereof, in one or more tissues of the plant, produces the initiation, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment syntheses, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of apical dominance and/or the delay of senescence, amongst other characteristics.

Moreover, the inventors have discovered further that additional effects which are not apparently related to the modification of cytokinin and/or gibberellin metabolism in the plant, can be produced by the ectopic expression of Cdc25 substrates or modified Cdc25 substrates in the plant. In particular, the inventors found that, by ectopically-expressing a substrate of Cdc25 or a modified substrate of Cdc25, and, in particular, Cdc2a or a homologue, analogue or derivative thereof, operably under the control of a regulatable plant-expressible promoter sequence, such as, for example, a constitutive promoter sequence, the transformed plants exhibited increased growth

and vigour; increased total biomass; increased cell number; reduced flowering time; increased branching; increased inflorescence formation; and increased seed set and/or increased seed yield and/or increased seed size.

The aforementioned attributes are of great agricultural and horticultural value since increased growth reduces the time-to-harvest of crop plants. This improvement is of considerable value in the case of forage crops. Furthermore, increased branching and increased plant vigour can be expected to have flow-on effects in terms of increased floral initiation, increased fruiting and higher seed yields. Such improvements are clearly of value to humans, because seed proteins are of high nutritional value to both humans and livestock.

Cyclin-dependent kinases, including Cdc25 and its substrates, such as, for example, Cdc2a, are intracellular proteins, which, unlike exogenously-applied cytokinin or cytokinin produced by ectopic expression of *ipt* or *rolC* genes, will only exert a localised effect at the site of protein synthesis. As a consequence, the present invention may be applied to the modification of a wide range of biochemical, morphological and phenotypic characteristics in plants, depending upon the promoter sequence selected to regulate ectopic expression of the introduced Cdc2a-encoding genetic sequence or a homologue, analogue or derivative thereof.

For example, one or more cytokinin-mediated and/or gibberellin-mediated effects are effectively modified using regulatable plant-operable promoter sequences selected from the group consisting of: cell-specific promoter sequences; tissue-specific promoter sequences; organ-specific promoter sequences; and cell cycle specific gene promoter sequences, such that the expression is targeted specifically to certain cells, tissues or organs of the transformed plants. Similarly, the modification of other characteristics in the plant can be performed using a constitutive promoter sequence. However, the present invention is not limited by the nature of the promoter used and the selection of promoter is clearly a preferred embodiment of the present invention.

Accordingly, one aspect of the invention provides a method of modifying plant morphology and/or biochemistry and/or physiology comprising expressing in particular cells, tissues or organs of a plant, a genetic sequence encoding a cyclin-dependent kinase (CDK) protein, and, in particular a substrate of Cdc25 or a modified substrate of Cdc25, operably in connection with a plant-operable promoter sequence.

Expression of the CDK is preferably carried out by introducing an isolated nucleic acid molecule comprising the CDK-encoding nucleotide sequence into a cell, tissue or organ of the plant, regenerating plant tissue or whole plants therefrom and then culturing those plant parts or whole plants under conditions suitable for activity of the promoter sequence to which said nucleotide sequence is operably connected.

Preferably, the genetic sequence encoding the cyclin-dependent kinase protein is placed operably under the control of a plant-expressible promoter sequence selected from the list comprising strong constitutive promoter sequences, cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences, cell-cycle-specific promoter sequences, and inducible promoter sequences (both pathogen-inducible and environmentally-inducible promoters are contemplated herein). The present invention further encompasses the use of a promoter sequence in a gene construct wherein an excisable genetic element is inserted into said construct so as to inactivate expression of the CDK protein during transformation and regeneration steps. According to this embodiment, excision of the excisable genetic element in the regenerated plant or progeny derived therefrom facilitates ectopic expression of the CDK protein. Methods to induce excision of such genetic elements are known to those skilled in the art. The excisable genetic element may be an autonomous or non-autonomous genetic element.

In a particularly preferred embodiment of the invention, the substrate of Cdc25 or a modified substrate of Cdc25 is a Cdc2a polypeptide or modified Cdc2a polypeptide, and more preferably a Cdc2a polypeptide or modified Cdc2a polypeptide of plants, such as, but not limited to, the *A. thaliana* Cdc2a polypeptide or a biologically-active

homologue, analogue or derivative thereof. The present invention clearly contemplates the use of functional homologues of nucleotide sequences encoding Cdc2a and, in particular, nucleotide sequences encoding a non-phosphorylateable, yet biologically-active, Cdc2a polypeptide, for example a Cdc2a polypeptide wherein threonine-14 and/or tyrosine-15 have been substituted with a non-phosphorylateable residue.

Preferred embodiments of the invention relate to the modification of one or more aspects of plant morphology and architecture by the ectopic expression of a Cdc25 substrate or modified Cdc25 substrate therein, including, but not limited to, increased growth and vigour and/or increased total biomass and/or increased cell number and/or modified flowering time and/or reduced branching and/or increased inflorescence formation and/or increased seed set and/or cytokinin-mediated effects and/or gibberellin-mediated effects.

With respect to cytokinin-mediated effects, the present invention clearly contemplates the broad application of the inventive method to the modification of a range of cellular processes, including but not limited to the modification of the initiation, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment syntheses, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of apical dominance and/or the delay of senescence.

With respect to gibberellin-mediated effects, the present invention clearly contemplated the broad application of the inventive method to the modification of a range of cellular processes, including but not limited to a modification of the inhibition, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment syntheses, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of apical dominance and/or the delay of

senescence.

In general applications of the invention, Cdc2a or a non-phosphorylateable Cdc2a protein is expressed in a whole plant under control of a regulatable promoter sequence, in particular a constitutive promoter sequence, such as, for example, the CaMV 35S promoter sequence, to increase growth and vigour and/or increase total biomass and/or increase cell number and/or modify flowering time and/or increase branching and/or increase inflorescence formation and/or increased seed set and/or seed size and/or seed yield.

In one preferred embodiment of the present invention, a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, is expressed operably under the control of a promoter derived from a stem-expressible gene, to increase the strength and thickness of a plant stem to confer improved stability and wind-resistance on the plant.

In another preferred embodiment of the present invention, a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, is expressed in a tuber-forming plant operably under the control of a promoter derived from a stem-expressible gene or tuber-expressible gene, to increase improve tuber production in the plant.

In another embodiment of the present invention, a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, is expressed in a tree crop plant such as, but not limited to, *Eucalyptus spp.* or *Populus spp.,* operably under the control of a promoter derived from a gene that is expressed in vascular tissue and/or cambium cells, to increase lignin content therein.

Without being bound by any theory or mode of action, the ectopic expression of a CDK under control of a promoter that is operably in vascular tissue and preferably, in cambial cells, produces thick-stemmed plants and a higher ratio of vascular tissue-to

-pith cells within the stem thereby resulting in more lignin production and a modified ratio of spring wood to late wood. Within the vascular tissue, cambial cells contain the highest levels of auxins and are therefore the preferential tissue for CDK overproduction.

In yet another preferred embodiment of the present invention, a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, is expressed operably under the control of a promoter derived from a seed-expressible gene, to increase seed production in plants, in particular to increase seed set and seed yield. More preferably, the promoter is operable in the endosperm of the seed, in which case the combination of the cell cycle-control protein and endosperm-expressible promoter provides the additional advantage of increasing the grain size and grain yield of the plant.

In yet another preferred embodiment of the present invention, a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, is expressed operably under the control of a promoter derived from a meristem-expressible gene or a shoot-expressible gene or a root-expressible gene, to reduce apical dominance and/or to promote bushiness of the plant and/or to increase or enhance the production of lateral roots and/or alter leaf shape.

In still another preferred embodiment of the present invention, a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, is expressed operably under the control of a promoter derived from a leaf-expressible gene, to alter leaf shape and/or to prevent or delay or otherwise reduce leaf chlorosis and/or leaf necrosis.

In a further preferred embodiment of the present invention, a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, is expressed under the control of a promoter that is operative in

meristem tissue of grain crops, to stimulate cell division in the intercalary meristem of the youngest stem internode and produce greater elongation of the stem and/or to generate a more extensive photosynthetic canopy.

The present invention may be applied to the selection of any cell, tissue, organ or whole organism that exhibits cytokinin-mediated and/or gibberellin-mediated morphological characteristics and/or biochemical characteristics and/or physiological characteristics, from a background of cells, tissues, organs or whole organisms that do note exhibit such characteristics.

A second aspect of the invention provides a gene construct or vector comprising a nucleotide sequence that encodes a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a non-phosphorylateable Cdc2a protein, operably under the control of a plant-expressible promoter sequence selected from the group consisting of:

(i) a plant-expressible cell-specific promoter sequence;

(ii) a plant-expressible tissue-specific promoter sequence;

(iii) a plant-expressible organ-specific promoter sequence;

(iv) a plant expressible inducible promoter sequence;

(v) a plant-expressible constitutive promoter sequence, wherein the nucleotide sequence encoding said substrate or modified substrate, and the plant-expressible constitutive promoter sequence, are integrated into an excisable genetic element;

(vi) a plant-expressible constitutive promoter sequence, wherein the nucleotide sequence encoding said substrate or modified substrate and said promoter sequence are such that expression of said substrate or modified substrate is capable of being modulated by an excisable genetic element; and

(vii) a plant-expressible cell cycle specific gene promoter sequence.

Preferably, the gene construct or vector according to this aspect of the invention is suitable for expression in a plant cell, tissue, organ or whole plant and more preferably,

the subject gene construct or vector is suitable for introduction into and maintenance in a plant cell, tissue, organ or whole plant.

A third aspect of the invention extends to those plant cells, tissues, organs and whole plants that have been transformed or transfected with a gene construct of the invention and/or which exhibit one or more modified phenotypic characteristics as described herein by virtue of the ectopic expression of a substrate of Cdc25 or a modified substrate of Cdc25.

For example, this aspect of the invention relates to those transformed plants that have been produced in accordance with the invention described herein and exhibit one or more plant morphological, biochemical, genetic, or physiological characteristics selected from the group consisting of: (i) cytokinin-mediated and/or gibberellin-mediated characteristics, such as, for example, modified stem length, tuber formation, lignin content, seed set and yield, bushiness, lateral root production, nitrogen fixation, leaf chlorosis, and leaf necrosis; (ii) increased growth and vigour; (iii) increased total biomass; (iv) increased cell number; (v) modified flowering time; (vi) increased branching; (vii) increased inflorescence formation; and (viii) increased seed set and/or seed size and/or increased seed yield; amongst others.

Transformed plants are preferably produced by the method of introducing into a plant cell, tissue or organ a nucleotide sequence capable of expressing a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, Cdc2a or a modified, non-phosphorylatable Cdc2a protein, and regenerating a whole plant therefrom, and more preferably, wherein the transformation procedure is performed without the application of exogenous cytokinin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photographic representation showing the stimulation of growth in *A. thaliana* plants that are homozygous at a single locus for the introduced plant-expressible *A. thaliana Cdc2aAt* gene, placed operably under control of the CaMV 35S

promoter sequence. **Panel a** shows genetically-untransformed plants. **Panel b** shows clonal variants of a representative transformed homozygous plant line. Plants were grown photosynthetically in containers permeable to atmospheric gases on Murashige and Skoog (1962) inorganic medium.

Figure 2 is a photographic representation of a western blot showing increased Cdc2a protein levels in *A. thaliana* plants that are homozygous at a single locus for the introduced plant-expressible *A. thaliana Cdc2aAt* gene, placed operably under control of the CaMV 35S promoter sequence.

Figure 3 is a photographic representation showing the presence of the introduced *Cdc2aA14F15* gene, operably in connection with the CaMV 35S promoter sequence, in the genomic DNA of transformed plants, as detected by PCR amplification. Lane 1, DNA size standards; Lanes 2 and 5, control PCR reactions with no plant DNA added; Lanes 3 and 4, PCR reactions comprising untransformed plant DNA; Lanes 6 and 7, PCR reactions comprising DNA derived from transgenic plant lines. Amplification reactions were performed using the primers indicated at the bottom of the Figure, as follows:

Primer 1: (WT 5'):	5′-GTTGAGAAGATTGGTGAAGGAACTTA- 3′;
Primer 2: (Cdc2aA14F15 5'):	5′-CCAAGATCCTTGAAGTATTCATGCTCC-3′;
Primer 3: (common 3'):	5′-GTTGAGAAGATTGGTGAAGGAGCTTT-3′.

The arrow indicates the position of an expected 856 bp amplification product derived from the modified *Cdc2a* gene.

Figure 4 is a photographic representation showing the stimulation of growth in *A. thaliana* plants that are homozygous at a single locus for the introduced plant-expressible *A. thaliana Cdc2aAtA14F15* gene (i.e. expressing a modified non-phosphorylateable Cdc2a protein), placed operably under control of the CaMV 35S promoter sequence. **Panel a** shows genetically-untransformed plants. **Panels b, c and d** show the representative transformed homozygous plant lines 1B1C1, 1B1D4, and 1C2C5, respectively. Transformed plants have roots and shoots that grow more

rapidly than un-transformed plants, and have more highly-branched roots than untransformed plants. Plants were grown photosynthetically in containers permeable to atmospheric gases on Murashige and Skoog (1962) inorganic medium.

Figure 5 is a photographic representation of a western blot showing increased Cdc2a protein levels in *A. thaliana* plant lines that are homozygous at a single locus for the introduced plant-expressible *A. thaliana Cdc2aAtA14F15* gene, placed operably under control of the CaMV 35S promoter sequence.

Figure 6 is a photographic representation showing the unmodified cell size in the lower leaf epidermis of transgenic *A. thaliana* plants that are homozygous at a single locus for an introduced *A. thaliana Cdc2aAtA14F15* gene. **Panel a,** wild type non-transformed plants. **Panels b, c and d** show the transformed lines 1BCA, 1B1D4 and 1C2C5, respectively. The interlocking perimeters and size of wild type epidermal cells seen in panel a are not altered in the transgenic plants, although they have grown more rapidly, by more rapid cell formation. Growth of the transformed plants was due to increased cell division and not a greater cell size than that of the untransformed plants.

Figure 7 is a photographic representation showing the presence of the *Cdc2aAtA14F15* transgene in *A. thaliana* plants, and enhanced growth of plants expressing the transgene. Panels a and B show the inherited *Cdc2aAtA14F15* transgene in F2 plants obtained by self-fertilisation of the primary transformant line YF-D, and the enhanced growth of those plants. Panels C and D show the presence of the *Cdc2aAtA14F15* transgene in the transgenic line YF-E, and the enhanced growth effects observed in plants expressing the transgene. Panels a and C show Western blots of *A. thaliana* protein probed with an antibody that is specific for the 34 kDa *A. thaliana* Cdc2a polypeptide. Panels B and D show total protein transferred to PVDF membranes. Lanes shown contain protein from *A. thaliana* plants having defined phenotypes as follows: **1**, large (big) transgenic plants; **2**, medium (mid)-sized transgenic plants; **3**, small transgenic plants; and **4**, non-transformed (WT) plants.

Briefly, 50 µg total protein from plants was subjected to SDS/PAGE, transferred to PVDF membranes and stained (panels B, D), then de-stained and probed with anti-At Cdc2a-serum (panels a, C).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the invention provides a method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, a cell cycle control protein comprising a substrate of Cdc25 or a modified substrate of Cdc25 operably under the control of a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences, and cell cycle-specific promoter sequences.

The word "modify" or variations such as "modifying" or "modified" as used herein with reference to any specified integer or group of integers shall be taken to indicate that said integer is altered by the performance of one or more steps pertaining to the invention described herein, compared to said integer in the absence of such performance.

Accordingly, by "modifying one or more plant morphological and/or biochemical and/or physiological characteristics" is meant that one or more morphological and/or biochemical and/or physiological characteristics of a plant is altered by the performance of one or more steps pertaining to the invention described herein.

"Plant morphology" or the term "plant morphological characteristic" or similar term will be understood by those skilled in the art to refer to the external appearance of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, colour, texture, arrangement, and patternation of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, stem, leaf, shoot, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, sieve

element, phloem or vascular tissue, amongst others.

As will be known to those skilled in the art, plants may modify one or more plant morphological characteristics in response to the external stimuli, such as, for example, a plant pathogenic infection, or an external stress (drought, flooding, salt stress, dehydration, heavy metal contamination, mineral deficiency, etc). Accordingly, for the present purpose, it shall be understood that a plant morphological characteristic that has been modified in response to one or more external stimuli is within the scope of the inventive method described herein, notwithstanding that the imposition of said external stimuli is not an essential feature of the present invention.

"Plant biochemistry" or the term "plant biochemical characteristic" or similar term will be understood by those skilled in the art to refer to the metabolic and catalytic processes of a plant, including primary and secondary metabolism and the products thereof, including any small molecules, macromolecules or chemical compounds, such as but not limited to starches, sugars, proteins, peptides, enzymes, hormones, growth factors, nucleic acid molecules, celluloses, hemicelluloses, calloses, lectins, fibres, pigments such as anthocyanins, vitamins, minerals, micronutrients, or macronutrients, that are produced by plants.

"Plant physiology" or the term "plant physiological characteristic" or similar term will be understood to refer to the functional processes of a plant, including developmental processes such as growth, expansion and differentiation, sexual development, sexual reproduction, seed set, seed development, grain filling, asexual reproduction, cell division, dormancy, germination, light adaptation, photosynthesis, leaf expansion, fibre production, secondary growth or wood production, amongst others; responses of a plant to externally-applied factors such as metals, chemicals, hormones, growth factors, environment and environmental stress factors (eg. anoxia, hypoxia, high temperature, low temperature, dehydration, light, daylength, flooding, salt, heavy metals, amongst others), including adaptive responses of plants to said externally-applied factors.

Preferably, the ectopic expression of a Cdc25 substrate or modified Cdc25 substrate protein produces a wide range of desirable phenotypes in the plant, selected from the group consisting of:(i) enhanced growth and/or enhanced vigour of the plant; (ii) increased total biomass of the plant; (iii) increased cell number; (iv) reduced flowering time; (v) increased inflorescence formation; (vi) reduced time to seed set (vii) enhanced seed set; (viii) enhanced seed size; (ix) enhanced grain yield; (x) enhanced stem strength; (xi) enhanced stem thickness; (xii) enhanced stem stability; (xiii) enhanced wind-resistance of the stem; (xiv) enhanced tuber formation; (xv) enhanced tuber development; (xvi) increased lignin content; (xvii) enhanced ploidy of the seed; (xviii) enhanced endosperm size; (xix) reduced apical dominance; (xx) increased bushiness; (xxi) enhanced lateral root formation; (xxii) enhanced rate of lateral root production; (xxiii) enhanced nitrogen-fixing capability; (xxiv) enhanced nodulation or nodule size; (xxv) reduced or delayed leaf chlorosis; (xxvi) reduced or delayed leaf necrosis; (xxvii) partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions; (xxviii) enhanced endoreplication; (xxix) enhanced endoreduplication, including enhanced endoreduplication in the seed; and (xxx) enhanced cell expansion.

More preferably, the plant morphological, biochemical or physiological characteristic which is modified is a cytokinin-mediated or a gibberellin-mediated characteristic selected from the group consisting of: (i) enhanced stem thickness; (ii) enhanced stem stability; (iii) enhanced wind-resistance of the stem; (iv) enhanced tuber formation; (v) enhanced tuber development; (vi) increased lignin content; (vii) enhanced seed set; (viii) enhanced seed production; (ix) enhanced grain yield; (x) enhanced ploidy of the seed; (xi) enhanced endosperm size; (xii) reduced apical dominance; (xiii) increased bushiness; (xiv) enhanced lateral root formation; (xv) enhanced rate of lateral root production; (xvi) enhanced nitrogen-fixing capability; (xvii) enhanced nodulation or nodule size; (xviii) reduced or delayed leaf chlorosis; (xix) reduced or delayed leaf necrosis; (xx) partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions; (xxi) enhanced endoreplication and/or enhanced endoreduplication; and (xxii) enhanced cell expansion.

According to this embodiment of the present invention, expression of the cell cycle control protein preferably leads to the initiation, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of apical dominance and/or the delay of senescence

In an alternative preferred embodiment, the plant morphological, biochemical or physiological characteristic which is modified is selected from the group consisting of: (i) plant growth and vigour; (ii) total biomass; (iii) cell number; (iv) flowering time; (v) branching; (vi) inflorescence formation; and (vii) seed set and/or seed yield and/or seed size. According to this embodiment of the present invention, expression of the cell cycle control protein preferably leads to increased plant productivity such as, for example, increased growth and vigour; increased total biomass; increased cell number; reduced flowering time; increased branching; increased inflorescence formation; and increased seed set and seed size.

The word "express" or variations such as "expressing" and "expression" as used herein shall be taken in their broadest context to refer to the transcription of a particular genetic sequence to produce sense or antisense mRNA or the translation of a sense mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA transcript, the word "express" or variations such as "expressing" and "expression" may also be construed to indicate the combination of transcription and translation processes, with or without subsequent post-translational events which modify the biological activity, cellular or sub-cellular localization, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

Accordingly, increased expression of a cell cycle control protein, such as a Cdc25 substrate or modified Cdc25 substrate, may exert its effect upon the morphology, biochemistry or physiology of the cell at the transcriptional or post-transcriptional level.

The term "cell cycle" as used herein shall be taken to include the cyclic biochemical and structural events associated with growth and with division of cells, and in particular with the regulation of the replication of DNA and mitosis. Cell cycle includes phases called: G0 (gap 0), G1 (gap 1), DNA replication (S), G2 (gap 2), and mitosis including cytokinesis (M). Normally these four phases occur sequentially. However, the cell cycle also includes modified cycles such as endomitosis, acytokinesis, polyploidy, polyteny, endopolyploidisation and endoreduplication or endoreplication.

The term "cell cycle interacting protein", "cell cycle protein", or "cell cycle control protein" as denoted herein means a protein which exerts control on or regulates or is required for the cell cycle or part thereof of a cell, tissue, organ or whole organism and/or DNA replication. It may also be capable of binding to, regulating or being regulated by cyclin dependent kinases or their subunits. The term also includes peptides, polypeptides, fragments, variant, homologues, alleles or precursors (eg preproproteins or preproteins) thereof.

Cell cycle control proteins and their role in regulating the cell cycle of eukaryotic organisms are reviewed in detail by John (1981) and the contributing papers therein (Norbury and Nurse 1992; Nurse 1990; Ormond and Francis 1993) and the contributing papers therein (Doerner *et al.* 1996; Elledge 1996; Francis and Halford 1995; Francis *et al.* 1998; Hirt *et al.* 1991; Mironov *et al.* 1999) which are incorporated herein by way of reference.

The term "cell cycle control gene" refers to any gene or mutant thereof which exerts positive or negative control on, or is required for, chromosomal DNA synthesis, mitosis (preprophase band, nuclear envelope, spindle formation, chromosome condensation, chromosome segregation, formation of new nuclei, formation of phragmoplast, etc) meiosis, cytokinesis, cell growth, or endoreduplication. The term "cell cycle control gene" also includes any and all genes that exert control on a cell cycle protein as hereinbefore defined, including any homologues of CDKs, cyclins, E2Fs, Rb, CKI, Cks, cyclin D, cdc25, Wee1, Nim1, MAP kinases, etc. Preferably, a cell cycle control gene will exert such regulatory control at the post-translation level, via interactions involving

the polypeptide product expressed therefrom.

More specifically, cell cycle control genes are all genes involved in the control of entry and progression through S phase. They include, not exclusively, genes expressing "cell cycle control proteins" such as cyclin dependent kinases (CDK), cycline dependent kinase inhibitors (CKI), D, E and a cyclins, E2F and DP transcription factors, pocket proteins, CDC7/DBF4 kinase, CDC6, MCM2-7, Orc proteins, cdc45, components of SCF ubiquitin ligase, PCNA, and DNA-polymerase, amongst others.

The term " cell cycle control protein" includes cyclins a, B, C, D and E, including CYCA1;1, CYCA2;1, CYCA3;1, CYCB1;1, CYCB1;2, CYCB2;2, CYCD1;1, CYCD2;1. CYCD3;1, and CYCD4;1 (Evans *et al.* 1983; Francis *et al.* 1998; Labbe *et al.* 1989; Murray and Kirschner 1989; Renaudin *et al* 1996; Soni *et al* 1995; Sorrell *et al* 1999; Swenson *et al* 1986); cyclin dependent kinase inhibitor (CKI) proteins such as ICK1 (Wang *et al* 1997), FL39, FL66, FL67 (PCT/EP98/05895), Sic1, Far1, Rum1, p21, p27, p57, p16, p15, p18, p19 (Elledge 1996; Pines 1995a,b), p14 and p14ARF; p13suc1 or CKS1At (De Veylder *et al* 1997; Hayles and Nurse 1986) and nim-1 (Russell and Nurse 1987a; Russell and Nurse 1987b; Fantes 1989; Russell and Nurse 1986; Russell and Nurse 1987a; Russell and Nurse 1987b) homologues of Cdc2 such as Cdc2MsB (Hirt *et al* 1993) CdcMs kinase (Bögre *et al* 1997) cdc2 T14Y15 phosphatases such as Cdc25 protein phosphatase or p80cdc25 (Bell *et al* 1993; Elledge 1996; Kumaghi and Dunphy 1991; Russell and Nurse 1986) and Pyp3 (Elledge 1996) cdc2 protein kinase or p34cdc2 (Colasanti *et al* 1991; Feiler and Jacobs 1990; Hirt *et al* 1991; John *et al* 1989; Lee and Nurse 1987; Nurse and Bissett 1981; Ormond and Francis 1993) cdc2a protein kinase (Hemerly *et al* 1993) cdc2 T14Y15 kinases such as wee1 or p107wee1 (Elledge 1996; Russell and Nurse 1986; Russell and Nurse 1987a; Russell and Nurse 1987a; Sun *et al* 1999) mik1 (Lundgren *et al* 1991) and myt1 (Elledge 1996); cdc2 T161 kinases such as Cak and Civ (Elledge 1996); cdc2 T161 phosphatases such as Kap1 (Elledge 1996); cdc28 protein kinase or p34cdc28 (Nasmyth 1993; Reed *et al.* 1985) p40MO15 (Fesquet *et al* 1993; Poon *et al.* 1993) chk1 kinase (Zeng *et al* 1998) cds1 kinase (Zeng *et al* 1998) growth associated H1 kinase (Labbe *et al* 1989; Lake and Salzman 1972; Langan 1978; Zeng

et al 1998) MAP kinases described by (Binarova *et al* 1998; Bögre *et al* 1999; Calderini *et al* 1998; Wilson *et al* 1999).

Other cell cycle control proteins are involved in cyclin D-mediated entry of cells into G1 from G0 include pRb (Xie *et al.*, 1996; Huntley *et al.*, 1998), E2F, RIP, MCM7, and the pRb-like proteins p107 and p130.

Other cell cycle control proteins are involved in the formation of a pre-replicative complex at one or more origins of replication, such as, but not limited to, ORC, CDC6, CDC14, RPA and MCM proteins or in the regulation of formation of this pre-replicative complex, such as, but not limited to, the CDC7, DBF4 and MBF proteins.

For the present purpose, the term "cell cycle control protein" shall further be taken to include any one or more of those proteins that are involved in the turnover of any other cell cycle control protein, or in regulating the half-life of said other cell cycle control protein. The term "protein turnover" is to include all biochemical modifications of a protein leading to the physical or functional removal of said protein. Although not limited to these, examples of such modifications are phosphorylation, ubiquitination and proteolysis. Particularly preferred proteins which are involved in the proteolysis of one or more of any other of the above-mentioned cell cycle control proteins include the yeast-derived and animal-derived proteins, Skp1, Skp2, Rub1, Cdc20, cullins, CDC23, CDC27, CDC16, and plant-derived homologues thereof (Cohen-Fix and Koshland 1997; Hochstrasser 1998; Krek 1998; Lisztwan *et al* 1998) and Plesse *et al* in (Francis *et al* 1998)).

For the present purpose, the term "cell cycle control genes" shall further be taken to include any one or more of those gene that are involved in the transcriptional regulation of cell cycle control gene expression such as transcription factors and upstream signal proteins. Additional cell cycle control genes are not excluded.

For the present purpose, the term "cell cycle control genes" shall further be taken to include any cell cycle control gene or mutant thereof, which is affected by

environmental signals such as for instance stress, nutrients, pathogens, or by intrinsic signals such as an animal mitogen or plant hormone (auxin, cytokinin, ethylene, gibberellic acid, abscisic acid and brassinosteroid).

The present invention clearly extends to specific equivalents, in particular the use of any Cdc25 substrate or modified substrate that possesses the biological activity of a cell cycle control protein, notwithstanding the fact that it may not be regulatable directly by Cdc25, such as, for example, a non-phosphorylateable Cdc25 substrate.

In the present context, the terms "substrate of Cdc25" and "Cdc25 substrate", or similar term, shall be taken to refer to any protein that is regulated directly or indirectly by Cdc25, and more particularly, any protein that is dephosphorylated by Cdc25, including, but not limited to cyclin-dependent kinases (CDKs). The CDK may be an A-type or a B-type CDK, and preferably, it is an A-type (or PSTAIRE-type) CDK, and more preferably, the CDK is a Cdc2 protein, in particular a Cdc2a protein. A Cdc2 protein, such as Cdc2a, is the key enzyme driving entry into S-phase and/or into mitosis (M-phase).

The inventors have also discovered that expression of a substrate of Cdc25, in particular a Cdc2 protein, in plants or plant cells can override the arrest of DNA replication (S phase) under growth-limiting conditions. Unexpectedly, the same process partially sustains endoreplication.

Preferred Cdc25 substrate proteins include Cdc2 and Cdc2a proteins.

Preferred modified Cdc25 substrates include non-phosphorylated and/or non-phosphorylateable Cdc25 substrate proteins, such as, for example non-phosphorylated or non-phosphorylateable Cdc2 proteins and/or non-phosphorylated or non-phosphorylateable Cdc2a proteins. In a particularly preferred embodiment, the modified Cdc25 substrate is the Cdc2aAt A14F15 polypeptide or a homologue, analogue or derivative thereof.

As used herein, unless specifically stated otherwise, or the context requires otherwise, reference to "a Cdc2 protein" shall be taken to include a reference to a Cdc2a protein, and, in particular, a reference to a Cdc2a polypeptide of plant origin, and including the *A. thaliana* Cdc2a polypeptide. Similarly, reference herein to "a modified Cdc2 protein" shall be taken to include a reference to a modified Cdc2a protein, and, in particular, a reference to a modified Cdc2a polypeptide of plant origin, and including the *A. thaliana* Cdc2aAtA14Y15 polypeptide.

The terms "modified substrate of Cdc25" and "modified Cdc25 substrate" shall be taken to refer to any isoform of a substrate of Cdc25 that mimics the effect of Cdc25 activity, in particular a non-phosphorylated form of Cdc2 that is produced by the dephosphorylation and/or inhibition of phosphorylation of a Cdc2 polypeptide. One example of a modified substrate of Cdc25 is a mutated A-type CDK, namely a Cdc2a protein, wherein threonine-14 and/or tyrosine-15 have been substituted with non-phosphorylatable residues, such as phenylalanine and/or alanine. Another example of a modified substrate of Cdc25 is a mutated B-type CDK, in particular Cdc2b, wherein threonine-14 and/or tyrosine-15 have been substituted with non-phosphorylatable residues, such as phenylalanine and/or alanine.

The term "modified substrate of Cdc25" also refers to a homologue, analogue or derivative of a substrate of Cdc25 that mimics the effect of Cdc25, such as, for example, a modified Cdc2a protein with increased activity compared to the wild-type Cdc2a protein, which preferably results in earlier switching from quiescence to active cycling and/or early entry of cells into S-phase and/or into mitosis (M-phase).

Accordingly, a modified substrate of Cdc25 encompasses any Cdc2 protein, including Cdc2a, or a homologue, analogue or derivative thereof which is non-phosphorylateable, or dephosphorylated, such that the modification removes the inhibitory effect of the phosphates on Cdc2 (or Cdc2a) and/or removes inhibition of Cdc2 (or Cdc2a) resulting from phosphorylation by wee-1 and/or mik-1, or otherwise increases Cdc2 (or Cdc2a) activity.

A particularly preferred modified substrate of Cdc25 is a modified Cdc2a protein wherein both threonine-14 and tyrosine-15 have been substituted with alanine and phenylalanine, respectively, to produce Cdc2aA14F15.

Preferably, a non-phosphorylatable form of Cdc2a is free of the phosphate at the tyrosine at position 15 (i.e. tyrosine-15 or Y15) and optionally (though not necessarily) free of the phosphate at threonine at position 14 (i.e. threonine-14 or T14).

In particular, the present inventors have shown that the ectopic expression of a modified substrate of Cdc25 which consists of the Cdc2aA14F15 polypeptide, produces growth effects and/or one or more cytokinin-like effects in the plants, especially increased meristem activity and outgrowth of lateral buds, leading to increased branching of plants that is increased relative to that observed following constitutive Cdc25 expression in plants. Without being bound by any theory or mode of action, these effects are presumably the result of the ectopically-expressed protein being both active and expressed at a high level.

Thus, the present invention encompasses *de novo* and/or increased expression of a substrate of Cdc25, or a modified substrate of Cdc25, such as a non-phosphorylatable Cdc2 or a modified Cdc2 protein with increased/earlier activity, or alternatively by raising Cdc2 activity by introducing additional copies of the Cdc2 gene to increase the amount of Cdc2 enzyme formed.

Without being bound by any theory or mode of action, the substitution or deletion of the phosphorylation sites of a Cdc2 protein mimics the effect of a constitutive phosphatase activity, such as the effect of the Cdc25 protein phosphatase ($p80^{Cdc25}$) activity. Moreover, the substitution or deletion of the phosphorylation sites of a Cdc2a protein further mimics the effect of down-regulated kinase activity, such as a down-regulation of the wee-1 kinase and/or mik-1. Those skilled in the art will know that the wee-1 and mik-1 kinase adds the inhibitory phosphate on threonine-14 and/or tyrosine 15. This is because phosphorylated protein will not be produced at high steady state concentrations in either the absence of phosphorylation or when phosphatase(s)

is(are) expressed at raised levels, or when kinase(s) is(are) expressed at lowered levels. Accordingly, the modified Cdc2a activity effects described herein can also be obtained, albeit only in part and without the benefits derived from increasing the amount of active Cdc2a protein, by the regulated expression of Cdc25. Alternatively, the modified Cdc2a activity effects described herein can also be obtained by down-regulating, or inhibiting, wee-1 and/or mik-1 kinase activity, such as, for example, by using antisense molecules, ribozymes, cosuppression molecules, gene targeting molecules, or gene silencing molecules, etc., which target the *wee-1* and/or *mik-1* genes or gene products, respectively.

The present invention involves providing *de novo* and/or increased expression of a substrate of Cdc25 or a modified substrate of Cdc25, such as, for example, a non-phosphorylatable Cdc2a or a modified Cdc2a with increased activity, or alternatively by raising Cdc2a activity by introducing additional copies of the Cdc2a gene to increase the amount of Cdc2a enzyme formed.

Those skilled in the art will be aware that biological activity of Cdc2a depends upon the presence of one or more cyclins in the cell, such as, for example, cyclin A and/or cyclin B. Accordingly, increased cyclin levels in plants to achieve the altered morphological, biochemical or physiological properties described herein are clearly within the scope of the present invention. In the use of cyclins to achieve these improvements, it is preferred that the ectopic expression of one or more cyclins in the plant accompanies the ectopic expression of Cdc2a or a modified substrate of Cdc25 described herein, in particular a modified Cdc2a protein such as Cdc2aA14F15.

In a particularly preferred embodiment, the present invention provides a method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, a Cdc2 protein or a homologue, analogue or derivative thereof, operably under the control of a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences, and cell cycle-specific promoter sequences.

"Homologues" of Cdc2 are those peptides. oligopeptides, polypeptides, proteins and enzymes which contain amino acid substitutions, deletions and/or additions relative to a non-mutant or wild-type Cdc2 polypeptide, without altering one or more of its cell cycle control properties.

To produce such homologues of Cdc2, amino acids present in Cdc2 can be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures or β-sheet structures, and so on.

Substitutional variants are those in which at least one residue in the Cdc2 amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the Cdc2 protein. Insertions can comprise amino– terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the Cdc2 sequence.

Amino acid variants of the Cdc2 polypeptide may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences

to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

In a preferred embodiment, the Cdc2 protein is the *A. thaliana* Cdc2a protein or a homologue, analogue or derivative thereof, in particular a non-phosphorylateable homologue, analogue or derivative.

More preferably, the Cdc2 homologue comprises a non-phosphorylateable Cdc2 protein, such as a Cdc2 protein wherein threonine-14 and/or tyrosine-15 is substituted with a non-phosphorylateable residue.

In a particularly preferred embodiment, the Cdc2 homologue is the Cdc2aA14F15 protein kinase (Hemerly *et al.,* 1995).

For the purposes of nomenclature, Cdc2aA14F15 comprises the amino acid substitutions T14⇒A14 and Y15⇒F15. The Cdc25 protein can dephosphorylate T14 and/or Y15 residues of a Cdc2 protein, to activate a Cdc2 protein kinase activity. In contrast, the wee-1 and/or mik-1 proteins phosphorylate T14 and/or Y15 residues of a Cdc2 protein to inactivate a Cdc2 protein kinase activity. Without being bound by any theory or mode of action, the Cdc2aA14F15 mutant protein is a non-phosphorylateable mutant that escapes regulation by phosphorylation and/or dephosphorylation.

In most plants for which CDK sequences have been identified, the positions of the said tyrosine-15 and threonine-14 are conserved, for example, as in the *A. thaliana* Cdc2a protein. Those skilled in the art will be aware of the possibility that, for example, during the course of evolution, the respective positions of these consensus amino acids have become altered. Such a shift may occur, for example, as a result of one or more deletions or additions at the N-terminus of the protein.

Accordingly, as used herein, the terms "tyrosine at position 15", or equivalent terms such as "tyrosine-15" or "Y15"; and "threonine at position 14" or equivalent terms such as "threonine-14" or "T14", refer herein to encompass the residues that are generally present at positions 15 and 14 of the respective CDK. The terms "tyrosine at position 15", or equivalent terms such as "tyrosine-15" or "Y15"; and "threonine at position 14" or equivalent terms such as "threonine-14" or "T14", refer further to tyrosine and threonine residues present in a CDK polypeptide at a different position to that which normally occurs, that possesses the inhibitory phosphorylatable characteristic of the Y15 and/or T14 residues with respect to CDK activity.

Determination of phosphorylation sites in CDKs corresponding to tyrosine at position 15 and threonine at position 14 of Cdc2a can be done for example by computer assisted identification of such sites in the amino acid sequence of a given CDK using eg BLAST2 (Basic Local Alignment Search Tool) which can be used to search for local sequence alignments. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologues. Phosphorylation sites can also be determined using anti-phospho-tyrosine and anti-phospho-threonine antibodies as described for instance by Zhang *et al.* (1996).

"Analogues" of a Cdc2 protein are defined as those peptides, oligopeptides, polypeptides, proteins and enzymes which are functionally equivalent to a Cdc2 protein.

Analogues of a Cdc2 protein include those Cdc25 substrates and modified Cdc25 substrates that comprise peptides, polypeptides, proteins, and enzymes that are capable of functioning in a plant cell and/or plant tissue and/or plant organ and/or whole plant to produce the same modified plant morphological and/or biochemical and/or physiological characteristics as the ectopic expression of a Cdc2 protein, including any functional homologues, mutants, derivatives, parts or fragments of Cdc2.

For example, any Cdc2 analogue may be substrate of Cdc25 or modified substrate of Cdc25, including the fission yeast Cdc2 protein, or a Cdc2 protein of plants, such as,

for example, a monocotyledonous plant or dicotyledonous plant species, and, in particular, tobacco, *A. thaliana*, maize, wheat, rice, barley, or other plant referred to herein.

Preferably, the Cdc2 analogue is a substrate of Cdc25 or a modified substrate of Cdc25 other than a cyclin B protein or a homologue or derivative thereof.

"Derivatives" of a Cdc2 protein are those peptides, oligopeptides, polypeptides, proteins and enzymes which comprise at least about five contiguous amino acid residues of a naturally-occurring Cdc2 polypeptide, in particular the *A. thaliana* Cdc2a polypeptide, but which retain activity in the induction of one or more of the morphological and/or biochemical and/or phenotypic characteristics that are capable of being modulated by expression of the full-length Cdc2 polypeptide as described herein.

A "derivative" of a Cdc2 protein may further comprise additional naturally-occurring, altered glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring Cdc2 polypeptide. Alternatively or in addition, a derivative may comprise one or more non-amino acid substituents compared to the amino acid sequence of a naturally-occurring Cdc2 polypeptide, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound thereto to facilitate its detection.

Other examples of recombinant or synthetic mutants and derivatives of the Cdc2 polypeptide include those incorporating single or multiple substitutions, deletions and/or additions therein, such as carbohydrates, lipids and/or proteins or polypeptides. Naturally-occurring or altered glycosylated or acylated forms of the Cdc2 polypeptide are also contemplated by the present invention. Additionally, homopolymers or heteropolymers comprising one or more copies of the Cdc2 polypeptide are within the scope of the invention, the only requirement being that such molecules retain activity in the induction of one or more of the morphological and/or biochemical and/or

phenotypic characteristics that are capable of being modulated by expression of the full-length Cdc2 polypeptide as described herein.

To effect expression of the Cdc25 substrate or modified Cdc25 substrate protein in a plant cell, tissue or organ, either the protein may be introduced directly to said cell, such as by microinjection means or alternatively, an isolated nucleic acid molecule encoding said protein may be introduced into the cell, tissue or organ in an expressible format.

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (anoxia, hypoxia, temperature, salt, light, dehydration, etc) or a chemical compound such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin) hormone (eg. gibberellin, auxin, cytokinin, glucocorticoid, etc), hormone analogue (iodoacetic acid (IAA), 2,4-D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

Preferably, expression of a Cdc25 substrate or modified Cdc25 substrate protein in a specific plant cell, tissue, or organ is effected by introducing and expressing an isolated nucleic acid molecule encoding said protein, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to said cell, tissue or organ, wherein said nucleic acid molecule is placed operably in connection with a plant-expressible promoter sequence.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or

without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

The term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a -10 box transcriptional regulatory sequences.

The term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive, glucocorticoid-responsive or dexamethasone-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible, glucocorticoid-inducible, or dexamethasone-inducible expression respectively, on said nucleic acid molecule.

In the context of the present invention, the promoter is a plant-expressible promoter sequence. By "plant-expressible" is meant that the promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, preferably a monocotyledonous or dicotyledonous plant cell and in particular a dicotyledonous plant cell, tissue, or organ. Accordingly, it is within the scope of the invention to include any promoter sequences that also function in non-plant cells, such as yeast cells, animal cells and the like.

The terms "plant-operable promoter sequence" and "promoter sequence operable in a plant" or similar term shall be taken to be equivalent to the term "plant-expressible

promoter sequence".

Preferably the promoter is a regulatable promoter sequence. In the present context, a "regulatable promoter sequence" is a promoter that is capable of conferring expression on a structural gene sequence in a particular cell, tissue, or organ or group of cells, tissues or organs of a plant, optionally under specific conditions. Accordingly, a regulatable promoter sequence may be a promoter sequence that confers expression on a gene to which it is operably connected in a particular location within the plant or alternatively, throughout the plant.

Included within the scope of such promoters are strong constitutive promoter sequences, cell-specific promoter sequences, inducible promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences, cell cycle-specific gene promoter sequences, and constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within an excisable genetic element.

The term "constitutive" will be known by those skilled in the art to indicate that expression is observed predominantly throughout the plant, albeit not necessarily in every cell, tissue or organ under all conditions. A strong constitutive promoter is one which confers a high level of ectopic expression on a structural gene to which it is operably connected, predominantly throughout the plant, albeit not necessarily in every cell, tissue or organ under all conditions.

The term "cell-specific" shall be taken to indicate that expression is predominantly in a particular plant cell or plant cell-type, albeit not necessarily exclusively in that plant cell or plant cell-type.

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular plant tissue or plant tissue-type, albeit not necessarily exclusively in that plant tissue or plant tissue-type.

Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular plant organ albeit not necessarily exclusively in that plant organ.

Similarly, the term "cell cycle specific" or similar shall be taken to indicate that expression is predominantly under control of the cell cycle, or capable of being cyclic such that it occurs in one or more phases of the cell cycle, albeit not necessarily in consecutive phases of the cell cycle, or in cycling cells.

Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental, chemical, environmental, or physical stimulus.

As will be apparent from the preceding description, the present invention does not require the exclusive expression of the cell cycle control protein in a cell, tissue or organ of a plant, in order to induce ectopic effects therein, however the expression of the cell cycle control protein should be regulatable such that it can be conferred in specific cells, tissues, or organs of the plant, or alternatively, induced under predetermined conditions, or during particular developmental stages of the plant.

Accordingly, in the case of expression conferred by certain constitutive promoter sequences, it is particularly preferred for the activity of the promoter sequence to be regulated by integrating the promoter sequence and cell cycle control gene in one or more excisable genetic elements.

As used herein, the term "an excisable genetic element" shall be taken to refer to any nucleic acid which comprises a nucleotide sequence which is capable of integrating into the nuclear, mitochondrial, or plastid genome of a plant, and subsequently being autonomously mobilised, or induced to mobilise, such that it is excised from the original integration site in said genome. By "autonomously mobilised" is meant that the genetic element is excised from the host genome randomly, or without the application of an external stimulus to excise. In performing the present invention, the genetic

element is preferably induced to mobilise, such as, for example, by the expression of a recombinase protein in the cell which contacts the integration site of the genetic element and facilitates a recombination event therein, excising the genetic element completely, or alternatively, leaving a "footprint", generally of about 20 nucleotides in length or greater , at the original integration site.

Preferably, the excisable genetic element comprises a transposable genetic element, such as, for example, *Ac, Ds, Spm,* or *En*, or alternatively, on or more loci for interaction with a site-specific recombinase protein, such as, for example, one or more *lox* or *frt* nucleotide sequences.

Known site-specific recombination systems, for example the cre/*lox* system and the flp/*frt* system which comprise a loci for DNA recombination flanking a selected gene, specifically *lox* or *frt* genetic sequences, combination with a recombinase, cre or flp, which specifically contacts said loci, producing site-specific recombination and deletion of the selected gene. In particular, European Patent No. 0228009 (E.I. Du Pont de Nemours and Company) published 29 April, 1987 discloses a method for producing site-specific recombination of DNA in yeast utilising the cre/*lox* system, wherein yeast is transformed with a first DNA sequence comprising a regulatory nucleotide sequence and a *cre* gene and a second DNA sequence comprising a pre-selected DNA segment flanked by two *lox* sites such that, upon activation of the regulatory nucleotide sequence, expression of the *cre* gene is effected thereby producing site-specific recombination of DNA and deletion of the pre-selected DNA segment. United States Patent No. 4,959,317 (E.I. Du Pont de Nemours and Company) filed 29 April 1987 and International Patent Application No. PCT/US90/07295 (E.I. Du Pont de Nemours and Company) filed 19 December, 1990 also disclose the use of the cre/*lox* system in eukaryotic cells.

A requirement for the operation of site-specific recombination systems is that the loci for DNA recombination and the recombinase enzyme contact each other *in vivo*, which means that they must both be present in the same cell. The prior art means for

excising unwanted transgenes from genetically-transformed cells all involve either multiple transformation events or sexual crossing to produce a single cell comprising *both* the loci for DNA recombination and the site-specific recombinase.

A "site-specific recombinase" is understood by those skilled in the relevant art to mean an enzyme or polypeptide molecule which is capable of binding to a specific nucleotide sequence, in a nucleic acid molecule preferably a DNA sequence, hereinafter referred to as a "recombination locus" and induce a cross-over event in the nucleic acid molecule in the vicinity of said recombination locus. Preferably, a site-specific recombinase will induce excision of intervening DNA located between two such recombination loci.

The terms "recombination locus" and "recombination loci" shall be taken to refer to any sequence of nucleotides which is recognized and/or bound by a site-specific recombinase as hereinbefore defined.

A number of different site specific recombinase systems can be used, including but not limited to the Cre/lox system of bacteriophage P1, the FLP/FRT system of yeast, the Gin recombinase of phase Mu, the Pin recombinase of *E.coli*, the PinB, PinD and PinF from *Shigella*, and the R/RS system of the psR1 plasmid. Some of these systems have already been used with high efficiency in plants, such as tobacco, and *A. thaliana*.

Preferred site-specific recombinase systems contemplated for use in the gene constructs of the invention, and in conjunction with the inventive method, are the bacteriophage P1 Cre/lox system, and the yeast FLP/FRT system. The site specific recombination loci for each of these two systems are relatively short, only 34 bp for the *lox* loci, and 47 bp for the *frt* loci.

In a most particularly preferred embodiment, however, the recombination loci are *lox* sites, such as *lox P, lox B, Lox L or lox R* or functionally-equivalent homologues, analogues or derivatives thereof. *Lox* sites may be isolated from bacteriophage or

bacteria by methods known in the art (Hoess *et al.*, 1982). It will also be known to those skilled in the relevant art that *lox* sites may be produced by synthetic means, optionally comprising one or more nucleotide substitutions, deletions or additions thereto.

The relative orientation of two recombination loci in a nucleic acid molecule or gene construct may influence whether the intervening genetic sequences are deleted or excised or, alternatively, inverted when a site-specific recombinase acts thereupon. In a particularly preferred embodiment of the present invention, the recombination loci are oriented in a configuration relative to each other such as to promote the deletion or excision of intervening genetic sequences by the action of a site-specific recombinase upon, or in the vicinity of said recombination loci.

The present invention clearly encompasses the use of gene constructs which facilitate the expression of a site-specific recombinase protein which is capable of specifically contacting the excisable genetic element, in conjunction with the gene constructs containing the cell cycle control protein-encoding gene. A single gene construct may be used to express both the site-specific recombinase protein and the cell cycle control protein, or alternatively, these may be introduced to plant cells on separate gene constructs.

For example, the recombinase gene could already be present in the plant genome prior to transformation with the gene construct of the invention, or alternatively, it may be introduced to the cell subsequent to transformation with the gene construct of the invention, such as, for example, by a separate transformation event, or by standard plant breeding involving hybridisation or cross-pollination. In one embodiment of the current invention, the recombinase gene is supplied to the transgenic plants containing a vector backbone sequence flanked by recombination sites by sexual crossing with a plant containing the recombinase gene in it's genome. Said recombinase can be operably linked to either a constitutive or an inducible promoter. The recombinase gene can alternatively be under the control of single subunit bacteriophage RNA

polymerase specific promoters, such as a T7 or a T3 specific promoter, provided that the host cells also comprise the corresponding RNA polymerase in an active form. Yet another alternative method for expression of the recombinase consists of operably linking the recombinase open reading frame with an upstream activating sequence fired by a transactivating transcription factor such as GAL4 or derivatives (US5801027, WO97/30164, WO98/59062) or the Lac repressor (EP0823480), provided that the host cell is supplied in an appropriate way with the transcription factor.

Alternatively, a substantially purified recombinase protein could be introduced directly into the eukaryotic cell, eg., by micro-injection or particle bombardment. Typically, the site-specific recombinase coding region will be operably linked to regulatory sequences enabling expression of the site-specific recombinase in the eukaryotic cell. In a preferred embodiment of the present invention, the site-specific recombinase sequences is operably linked to an inducible promoter.

Dual-specific recombinase systems can also be employed, which may employ a recombinase enzyme in conjunction with direct or indirect repeats of two different site-specific recombination loci corresponding to the dual-specific recombinase, such as that described in International Patent Publication No. WO99/25840.

As will be known to those skilled in the art, for recombination mediated by a transposon to occur, a pair of DNA sequences comprising inverted repeat transposon border sequences, flanking the excisable genetic element sequence, and a specific transposase enzyme, are required. The transposase catalyzes a recombination reaction only between two transposon border sequences.

A number of different plant-operable transposon/transposase systems can be used including but not limited to the *Ac/Ds* system, the *Spm* system and the *Mu* system. All of these systems are operable in *Zea mays*, and at least the *Ac/Ds* and the *Spm* system function in other plants.

Preferred transposon sequences for use in the gene constructs of the invention are the *Ds*-type and the *Spm*-type transposons, which are delineated by border sequences of only 11 bp and 13 bp in length, respectively.

As with the use of site-specific recombinase systems, the present invention clearly encompasses the use of gene constructs which facilitate the expression of a transposase enzyme which is capable of specifically contacting the transposon border sequence, in conjunction with the gene constructs containing thecell cycle control protein-encoding gene. A single gene construct may be used to express both the transposase and the cell cycle control protein, or alternatively, these may be introduced to plant cells on separate gene constructs.

For example, the transposase-encoding gene could already be present in the plant genome prior to transformation with the gene construct of the invention, or alternatively, it may be introduced to the cell subsequent to transformation with the gene construct of the invention, such as, for example, by a separate transformation event, or by standard plant breeding involving hybridisation or cross-pollination. Alternatively, a substantially purified transposase protein could be introduced directly into the eukaryotic cell, eg., by micro-injection or particle bombardment. Typically, the transposase coding region will be operably linked to regulatory sequences enabling expression of the transposase in the eukaryotic cell. In a preferred embodiment of the present invention, the transposase-encoding sequence is operably linked to an inducible promoter.

In the present context, transposon border sequences are organized as inverted repeats flanking the excisable genetic element. As transposons often re-integrate at another locus of the host's genome, segregation of the progeny of the hosts in which the transposase was allowed to act might be necessary to separate transformed hosts containing only the gene(s) of interest and transformed hosts containing only the cell cycle control protein-encoding gene.

Likewise, the site-specific recombinase gene or transposase gene present in the host's genome can be removed by segregation of the progeny of the hosts to separate transformed hosts containing only the gene(s) of interest and transformed hosts containing only the site-specific recombinase gene or transposase gene. Alternatively, said site-specific recombinase gene or transposase gene are included in the same or in a different excisable genetic element as thecell cycle control protein-encoding gene.

Notwithstanding the generality of the present invention, wherein the plant morphological, biochemical or physiological characteristic which is modified is a cytokinin-mediated or a gibberellin-mediated characteristic, it is preferred that the promoter is a cell-specific, tissue-specific, organ-specific, or cell cycle-specific promoter sequence. Wherein the plant morphological, biochemical or physiological characteristic which is modified is selected from the group consisting of: (i) plant growth and vigour; (ii) total biomass; (iii) cell number; (iv) flowering time; (v) branching; (vi) inflorescence formation; and (vii) seed set and/or seed size and/or seed yield, it is preferred that the promoter is a strong constitutive promoter sequence. However, as mentioned previously, the present invention is not to be limited by the choice of promoter sequence.

Preferably, the promoter selected for regulating expression of the cell cycle control protein in a plant cell, tissue or organ, will confer expression in a range of cell-types or tissue-types or organs, sufficient to produce the desired phenotype, whilst avoiding undesirable phenotypes produced in other cell-types or tissue-types or organs.

Those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression of a Cdc25 substrate or modified Cdc25 substrate, including a Cdc2 protein or a modified Cdc2 protein, such as, for example, the Cdc2a or modified Cdc2a polypeptides and homologues, analogues and derivatives thereof, using publicly-available or readily-available sources, such as those listed in Table 1.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence,

or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence.

A promoter is usually, but not necessarily, positioned upstream, or at the 5′-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates.

In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in gene constructs of the present invention include those listed in Table 1, amongst others. The promoters listed in Table 1 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

In an alternative embodiment, the promoter is a tissue-specific inducible promoter sequence, such as but not limited to a light-inducible *rbcs-1A* or *rbcs-3A* promoter, anoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987), hypoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987), and the temperature-inducible heat shock promoter. Such environmentally-inducible promoters are reviewed in detail by Kuhlemeier *et al.* (1987).

In an alternative embodiment, the promoter is a chemically-inducible promoter, such

as the 3-β- indoylacrylic acid-inducible *Tip* promoter; IPTG-inducible *lac* promoter; phosphate-inducible promoter; L-arabinose-inducible *araB* promoter; heavy metal-inducible metallothionine gene promoter; dexamethasone-inducible promoter; glucocorticoid-inducible promoter; ethanol-inducible promoter (Zeneca); the N,N-diallyl-2,2-dichloroacetamide-inducible glutathione-S-transferase gene promoter (Wiegand *et al.*, 1986); or any one or more of the chemically-inducible promoters described by Gatz *et al.* (1996;1998), amongst others.

In an alternative embodiment, the promoter is a wound-inducible or pathogen-inducible promoter, such as the phenylalanine ammonia lyase (PAL) gene promoter (Ebel *et al.*, 1984), chalcone synthase gene promoter (Ebel *et al.*, 1984) or the potato wound-inducible promoter (Cleveland *et al.*, 1987), amongst others.

In a further alternative embodiment, the promoter is a hormone-inducible promoter, such as the abscisic acid-inducible wheat 7S globulin gene promoter and the wheat Em gene promoter (Marcotte *et al.*,1988); an auxin-responsive gene promoter, such as, for example, the *SAUR* gene promoter, the *parAs* and *parAt* gene promoters(van der Zaal *et al.*, 1991; Gil *et al.*, 1994; Niwa *et al.*, 1994); or a gibberellin-inducible promoter such as the *Amy32b* gene promoter (Lanahan *et al.* 1992), amongst others.

In yet another alternative embodiment, the promoter is a cell cycle-specific gene promoter, such as the Cdc2a gene promoter sequence described by Chung and Parish (1995), or the PCNA gene promoter sequence described by Kosugi *et al.* (1991), and Kosugi and Ohashi (1997), amongst others.

In a further alternative embodiment, the promoter is a strong constitutive plant-expressible promoter sequence such as the CaMV 35S promoter sequence, CaMV 19S promoter sequence, the octopine synthase (OCS) promoter sequence, or nopaline synthase (NOS) promoter sequence (Ebert *et al.* 1987), amongst others.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, such sequences may be modified by the addition of nucleotide

sequences derived from one or more of the tissue-specific promoters listed in Table 1, or alternatively, nucleotide sequences derived from one or more of the above-mentioned tissue-specific inducible promoters, to confer tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1* promoter sequence, to confer anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.*, 1987). Such modifications can be achieved by routine experimentation by those skilled in the art.

In a particularly preferred embodiment of the present invention, there is provided a method of modifying a morphological or physiological characteristic of a plant selected from the group consisting of: (i) increased growth and vigour; (ii) increased total biomass; (iii) increased cell number; (iv) modified flowering time; (v) increased branching; (vi) increased inflorescence formation; and (vii) increased seed set comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of the CaMV 35S promoter sequence.

In a further particularly preferred embodiment of the present invention, there is provided a method of increasing the strength and/or thickness and/or stability and/or wind-resistance of a plant comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein, therein operably under the control of a stem-expressible promoter sequence.

Preferably, the stem-expressible promoter sequence is derived from the *rbcs-1A* gene, the *rbcs-3A* gene, the *AtPRP4* gene, the *T. bacilliform* virus gene, or the sucrose-binding protein gene set forth in Table 1, or a stem-specific or stem-expressible homologue, analogue or derivative thereof.

In another preferred embodiment of the present invention, there is provided a method of increasing tuber formation and/or development in a tuberous crop plant comprising

ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of a tuber-specific promoter sequence.

Preferably, the tuberous crop plant is potato and the tuber-specific promoter is the potato patatin gene promoter. Additional species and promoters are not excluded.

In another preferred embodiment of the present invention, there is provided a method of modifying the lignin content of a woody crop plant comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein, therein operably under the control of a cambium-specific or vascular-tissue-specific promoter sequence.

Preferably, the promoter is a cinnamoyl alcohol dehydrogenase (CAD) gene promoter, laccase gene promoter, cellulose synthase gene promoter and xyloglucan endotransglucosylase (XET) gene promoter sequences, amongst others. The *T. bacilliform* virus gene promoter and the sucrose-binding protein gene promoter are also useful for this application of the invention.

Preferred target plant species according to this embodiment are woody plants of economic/ agronomic value, in particular hardwood crop plants such as, but not limited to *Eucalyptus spp., Populus spp., Quercus spp., Acer spp., Juglans spp., Fagus spp., Acacia spp.,* or teak, amongst others. More preferably, this embodiment of the invention is applicable to modifying the lignin content of *Eucalyptus spp.,* in particular *E. globulus* and *E. robusta;* or *Quercus spp.,* in particular *Q. dentata, Q. ilex, Q. incana,* and *Q. robur; Acacia spp.,* in particular *a. brevispica, a. bussei, a. drepanolobium, a. nilotica, a. pravissima,* and *a. seyal; Acer spp.,* in particular *a. pseudoplatanus* and *a. saccharum*. Additional species are not excluded.

TABLE 1

EXEMPLARY PLANT-OPERABLE PROMOTERS FOR USE IN PERFORMING THE PRESENT INVENTION

I: CELL-SPECIFIC, TISSUE-SPECIFIC, AND ORGAN-SPECIFIC PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
α-amylase (Amy32b)	aleurone	Lanahan *et al.*, 1992; Skriver *et al.*, 1991
cathepsin β-like gene	aleurone	Cejudo *et al.*,1992.
Agrobacterium rhizogenes rolB	cambium	Nilsson *et al.*, 1997
PRP genes	cell wall	http://salus.medium.edu/mmg/tierney/html
AtPRP4	flowers	http://salus.medium.edu/mmg/tierney/html
chalene synthase (chsA)	flowers	Van der Meer *et al.*, 1990.
LAT52	anther	Twell *et al.*,1989
apetala-3	flowers	
chitinase	fruit (berries, grapes, etc)	Thomas *et al.* CSIRO Plant Industry, Urrbrae, South Australia, Australia; http://winetitles.com.au/gwrdc/ csh95-1.html
rbcs-3A	green tissue (eg leaf)	Lam *et al.*, 1990; Tucker *et al.*, 1992.
leaf-specific genes	leaf	Baszczynski *et al.*, 1988.
AtPRP4	leaf	http://salus.medium.edu/mmg/tierney/html
Pinus cab-6	leaf	Yamamoto *et al.*, 1994.
SAM22	senescent leaf	Crowell *et al.*, 1992.
R. japonicum nif gene	nodule	United States Patent No. 4, 803, 165
B. japonicum nifH gene	nodule	United States Patent No. 5, 008, 194
GmENOD40	nodule	Yang *et al.* 1993
PEP carboxylase (PEPC)	nodule	Pathirana *et al.*, 1992.

leghaemoglobin (Lb)	nodule	Gordon *et al.*, 1993.
Tungro bacilliform virus gene	phloem	Bhattacharyya-Pakrasi *et al.*, 1992.
sucrose-binding protein gene	plasma membrane	Grimes *et al.*, 1992.
pollen-specific genes	pollen; microspore	Albani *et al.*, 1990; Albani *et al.*, 1991
Zm13	pollen	Guerrero *et al.*, 1993
apg gene	microspore	Twell *et al.*, 1993
maize pollen-specific gene	pollen	Hamilton *et al.*, 1992.
sunflower pollen-expressed gene	pollen	Baltz *et al.*, 1992.
B. napus pollen-specific gene	pollen;anther; tapetum	Arnoldo *et al.*, 1992.
root-expressible genes	roots	Tingey *et al.*, 1987.
tobacco auxin-inducible gene	root tip	Van der Zaal *et al.*, 1991.
β-tubulin	root	Oppenheimer *et al.*, 1988.
tobacco root-specific genes	root	Conkling *et al.*, 1990.
B. napus G1-3b gene	root	United States Patent No. 5, 401, 836
SbPRP1	root	Suzuki *et al.*, 1993.
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
AtPRP4	leaves; flowers; lateral root primordia	http://salus.medium.edu/mmg/tierney/html
seed-specific genes	seed	Simon *et al.*, 1985; Scofield *et al.*, 1987; Baszczynski *et al.*, 1990.
Brazil Nut albumin	seed	Pearson *et al.*, 1992.
legumin	seed	Ellis *et al.*, 1988.
glutelin (rice)	seed	Takaiwa *et al.*, 1986; Takaiwa *et al.*, 1987.
zein	seed	Matzke *et al.*, 1990
napA	seed	Stalberg *et al.*, 1996.

wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2, 1989
wheat SPA	seed	Albani *et al.*,1997
wheat α, β, γ-gliadins	endosperm	EMBO 3:1409-15, 1984
barley ltr1 promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena *et al.*, 1998
blz2	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa *et al.*, 1998.
rice prolamin NRP33	endosperm	Wu *et al.*, 1998
rice α-globulin Glb-1	endosperm	Wu *et al.*, 1998
rice OSH1	embryo	Sato *et al.*,1996
rice α-globulin REB/OHP-1	endosperm	Nakase *et al.*,1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorgum γ-kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	embryo	Postma-Haarsma *et al.*, 1999
rice oleosin	embryo and aleurone	Wu et at,1998
sunflower oleosin	seed (embryo and dry seed)	Cummins *et al.*, 1992
LEAFY	shoot meristem	Weigel *et al.*, 1992.
Arabidopsis thaliana knat1	shoot meristem	Accession number AJ131822
Malus domestica kn1	shoot meristem	Accession number Z71981
CLAVATA1	shoot meristem	Accession number AF049870
stigma-specific genes	stigma	Nasrallah *et al.*, 1988; Trick *et al.*, 1990
class I patatin gene	tuber	Liu *et al.*, 1991.
PCNA rice	meristem	Kosugi *et al.*, 1991; Kosugi *et al.*, 1997.

Pea TubA1 tubulin	Dividing cells	Stotz *et al.*,1999
Arabidopsis cdc2a	cycling cells	Chung and Parish, 1995
Arabidopsis Rop1A	Anthers; mature pollen; pollen tube	Li *et al.*, 1998
Arabidopsis AtDMC1	Meiosis-associated	Klimyuk and James,1997
Pea PS-IAA4/5 and PS-IAA6	Auxin-inducible	Wong *et al.*, 1996
Pea farnesyltransferase	Meristematic tissues; phloem near growing tissues; light- and sugar-repressed	Zhou *et al.*, 1997
Tobacco (N. sylvestris) cyclin B1;1	Dividing cells / meristematic tissue	Trehin *et al.*, 1997
Catharanthus roseus Mitotic cyclins CYS (A-type) and CYM (B-type)	Dividing cells / meristematic tissue	Ito *et al.*, 1997
Arabidopsis cyc1At (=cyc B1;1) and cyc3aAt (A-type)	Dividing cells / meristematic tissue	Shaul *et al.*, 1996
Arabidopsis tef1 promoter box	Dividing cells / meristematic tissue	Regad *et al.*, 1995
Catharanthus roseus cyc07	Dividing cells / meristematic tissue	Ito *et al.*, 1994

II: EXEMPLARY CONSTITUTIVE PROMOTERS

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
Actin	constitutive	McElroy *et al.*, 1990
CAMV 35S	constitutive	Odell *et al.*, 1985
CaMV 19S	constitutive	Nilsson *et al.*, 1997
GOS2	constitutive	de Pater *et al.*,1992
ubiquitin	constitutive	Christensen *et al.*, 1992
rice cyclophilin	constitutive	Buchholz *et al.*, 1994
maize H3 histone	constitutive	Lepetit *et al.*, 1992
actin 2	constitutive	An *et al.*, 1996

III: EXEMPLARY STRESS-INDUCIBLE PROMOTERS

NAME	STRESS	REFERENCE
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang *et al.*, 1997
cor15a	cold	Hajela *et al.*, 1990
cor15b	cold	Wilhelm and Thomashow, 1993
cor15a (-305 to +78 nt)	cold, drought	Baker *et al.*, 1994
rd29	salt, drought, cold	Kasuga *et al.*, 1999
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros *et al.*, 1992. Marrs *et al.*, 1993. Schoffl *et al.*, 1989.
smHSP (small heat shock proteins)	heat	Waters *et al.*, 1996
wcs120	cold	Ouellet *et al.*, 1998
ci7	cold	Kirch *et al.*, 1997
Adh	cold, drought, hypoxia	Dolferus *et al.*, 1994
pwsi18	water: salt and drought	Joshee *et al.*, 1998
ci21A	cold	Schneider *et al.*, 1997
Trg-31	drought	Chaudhary *et al.*, 1996
osmotin	osmotic	Raghothama *et al.*, 1993
lapA	wounding, enviromental	WO99/03977 University of California/INRA

IV: EXEMPLARY PATHOGEN-INDUCIBLE PROMOTERS		
NAME	**PATHOGEN**	**REFERENCE**
RB7	Root-knot nematodes (Meloidogyne spp.)	US5760386 - North Carolina State University; Opperman *et al.*, 1994
PR-1, 2, 3, 4, 5, 8, 11	fungal, viral, bacterial	Ward *et al.*, 1991; Reiss and Bryngelsson, 1996; Lebel *et al.*, 1998; Melchers *et al.*, 1994; Lawton *et al.*, 1992
HMG2	nematodes	WO9503690 - Virginia Tech Intellectual Properties Inc .
Abi3	Cyst nematodes (Heterodera spp.)	unpublished
ARM1	nematodes	Barthels *et al.*, 1997; WO 98/31822 – Plant Genetic Systems
Att0728	nematodes	Barthels *et al.*, 1997; PCT/EP98/07761
Att1712	nematodes	Barthels *et al.*, 1997; PCT/EP98/07761
Gst1	Different types of pathogens	Strittmatter *et al.*, 1996
LEMMI	nematodes	WO 92/21757 – Plant Genetic Systems
CLE	geminivirus	PCT/EP99/03445 - CINESTAV
PDF1.2	Fungal including *Alternaria brassicicola* and *Botrytis cinerea*	Manners *et al.*, 1998
Thi2.1	Fungal – *Fusarium oxysporum f sp. matthiolae*	Vignutelli *et al.*, 1998
DB#226	nematodes	Bird and Wilson, 1994; WO 95.322888
DB#280	nematodes	Bird and Wilson, 1994; WO 95.322888
Cat2	nematodes	Niebel *et al.*, 1995
αTub	nematodes	Aristizabal *et al.* (1996), 8th International Congress on Plant-Microbe Interaction, Knoxville US B-29
sHSP	nematodes	Fenoll *et al.*, 1997
Tsw12	nematodes	Fenoll *et al.*, 1997
Hs1(pro1)	nematodes	WO 98/122335 - Jung
nsLTP	viral, fungal, bacterial	Molina *et al.*, 1993
RIP	viral, fungal	Tumer *et al.*, 1997

Without being bound by any theory or mode of action, the ectopic expression of Cdc2a or a variant thereof under control of a promoter that is operable in vascular tissue and preferably, in cambial cells, will produce thick-stemmed plants and a higher ratio of vascular tissue-to-pith cells within the stem, thereby resulting in more lignin production. Within the vascular tissue, cambial cells contain the highest levels of auxins and are therefore the preferential tissue for the overexpression of a Cdc2 protein or a modified Cdc2 protein.

In yet another preferred embodiment of the present invention, there is provided a method of increasing seed set and/or seed production and/or seed size and/or grain yield in a plant comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of a seed-specific promoter sequence.

Preferably, the seed-specific promoter is operable in the seeds of monocotyledonous plants, for example the barley *blz2* gene promoter, the barley *Amy32b* gene promoter, Cathepsin β-like gene promoter, wheat ADP-glucose pyrophosphorylase gene promoter, maize zein gene promoter, or rice glutelin gene promoter. In an alternative embodiment, the seed-specific promoter is operable in the seeds of dicotyledonous plant species, for example the legumin gene promoter, *napA* gene promoter, Brazil Nut albumin gene promoter, pea vicilin gene promoter and sunflower oleosin gene promoter, amongst others.

Those skilled in the art will be aware that grain yield in crop plants is largely a function of the amount of starch produced in the endosperm of the seed. The amount of protein produced in the endosperm is also a contributing factor to grain yield. In contrast, the embryo and aleurone layers contribute little in terms of the total weight of the mature grain. By virtue of being linked to cell expression and metabolic activity, endoreplication and endoreduplication are generally considered as an important factor

for increasing yield (Traas *et al* 1998). As grain endosperm development initially includes extensive endoreplication (Olsen *et al* 1999), enhancing, promoting or stimulating this process is likely to result in increased grain yield. Enhancing, promoting or stimulating cell division during seed development is an alternative way to increase grain yield. The present invention clearly encompasses the use of a Cdc25 substrate or modified Cdc25 substrate, such as a Cdc2 protein or modified Cdc2 protein, to modify endoreplucation and/or stimulate cell division.

Accordingly, in a preferred embodiment, a Cdc2-encoding gene, preferably the *Cdc2a* or *Cdc2aA14F15* gene, is placed operably in connection with a promoter that is operable in the endosperm of the seed, in which case the combination of the cell cycle-control protein and endosperm-expressible promoter provides the additional advantage of increasing the grain size and grain yield of the plant.

Endosperm-specific promoters that can be used to drive expression of a Cdc25 substrate or modified Cdc25 substrate protein have been identified. The components of the promoters responsible for specific expression have been identified (Grosset et al (1997) and are interchangeable between agriculturally important cereals (Olsen et al 1992; Russell and Fromm, 1997). Several promoters can be used, including the barley *blz2* gene promoter, the rice prolamin NRP33 promoter, the rice REB promoter, the zein (ZmZ27) gene promoter, the rice glutelin 1 gene (osGT1) promoter, the rice small subunit ADP-glucose pyrophosphorylase (osAGP) promoter, the maize granule-bound starch synthase (Waxy) gene (zmGBS) promoter surveyed by Russell and Fromm (1997), the Brazil Nut albumin gene promoter, and the pea vicilin gene promoter, amongst others. Promoters derived from those genes that are expressed in the endosperm during nuclear proliferation are also useful for driving expression of a Cdc25 substrate or modified Cdc25 substrate protein, such as, for example a Cdc2a protein or a homologue, analogue or derivative thereof, preferably Cdc2aA14F15. Promoters derived from those genes that are expressed in the endosperm at the stage when nuclear proliferation is ending could be ideal for extending this period.

In a preferred embodiment, the promoter sequence is derived from the wheat *END1* gene described by Doan *et al.* (1996).

A three way correlation exists between cytokinin level in the endosperm, the number of endosperm cells formed during seed development and grain size, in which cytokinin activates Cdc2a kinase or modified Cdc2a kinase to drive nuclear division. Accordingly, ectopic expression of the gene encoding a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein, in the endosperm enhances nuclear proliferation, resulting in increased grain size, without incurring the non-specific side effects that application of cytokinin or expression of the *ipt* gene would produce in the plant.

A further advantage of the present inventive approach is that the activity of cytokinin metabolising enzymes is circumvented by the ectopic expression of a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein, in the endosperm, thereby raising the activity of Cdc25 substrates or modified Cdc25 substrates therein. In cases where exogenous cytokinin is used to increase grain size and/or endosperm size, the elevated cytokinin levels and nuclear division in the grain are curtailed by an increase in the activities of cytokinin degrading enzymes, including cytokinin oxidase (Chatfield and Armstrong 1987; reviewed by Morris *et al* 1993).

In another preferred embodiment of the present invention, there is provided a method of inhibiting or reducing apical dominance or increasing the bushiness of a plant, comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of a meristem-specific promoter sequence or a stem-specific promoter sequence.

Without being bound by any theory or mode of action, increased cell division in the dormant lateral meristem of plants as a consequence of increased Cdc25 substrates or modified Cdc25 substrates therein results in a higher degree of branch formation in the plant, thereby alleviating auxin-induced apical dominance in the plant.

In another preferred embodiment of the present invention, there is provided a method of increasing lateral root production in a plant comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of a root-specific promoter sequence.

Preferred promoter sequences according to this embodiment of the present invention include any one of the root-expressible or root-specific promoters listed in Table 1 and in particular, the tobacco auxin-inducible gene promoter described by Van der Zaal *et al* (1991) that confers expression in the root tip of plants, in particular dicotyledonous plants.

In yet another preferred embodiment of the present invention, there is provided a method of increasing the nitrogen-fixing capability of a plant comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of a nodule-specific promoter sequence.

Preferred nodule-specific promoter sequences according to this embodiment of the present invention are listed in Table 1. Additional promoters that are suited for this purpose include the hemoglobin gene promoters derived from *Frankia spp., A. thaliana* or other plants.

In still another preferred embodiment of the present invention, there is provided a method of preventing or delaying or otherwise reducing leaf chlorosis and/or leaf necrosis in a plant comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of a leaf-specific promoter sequence.

Preferred promoters for use according to this embodiment of the present invention include the SAM22 promoter, *rbcs-1A* and *rbcs-3A* gene promoters listed in Table 1. The SAM22 gene promoter is particularly preferred in light of the developmental regulation of the SAM22 gene and its induction in senescent leaves.

In a further preferred embodiment of the present invention, *A. thaliana* Cdc2a or a homologue analogue or derivative thereof, in particular Cdc2aA14F15, is expressed in one of the specialised minority of plant tissues in which the activation of cell cycle progression that is generally contributed by cytokinin is in part performed by other hormones. An example of such a tissue is the youngest stem internode of cereal plants in which gibberellic acid stimulates cell division.

Accordingly, the present invention preferably provides a method of stimulating cell division in the intercalary meristem of the youngest stem internode to produce greater elongation of the stem and/or to generate a more extensive photosynthetic canopy of a plant comprising ectopically expressing a Cdc2 protein, preferably a modified Cdc2 protein, more preferably a modified Cdc2a protein or a homologue, analogue or derivative thereof, and, in particular, a Cdc2aA14F15 protein therein operably under the control of a meristem specific promoter sequence.

Without being bound by any theory or mode of action, increase in cell division in the intercalary meristem of the youngest stem internode as a consequence of increased

Cdc25 substrate or modified Cdc25 substrate activity therein results in greater vigour of the plant due to stem elongation and the production of a more extensive canopy. It is proposed that this leads to an increase in the plant's capacity to support grain production. The stimulatory effect of gibberellic acid application is thus obtained without side effects on flowering time and seed germination.

Preferred promoters for use according to this embodiment of the invention include meristem promoters listed in Table 1 and in particular the Proliferating Cell Nuclear Antigen (PCNA) promoter of rice described by Kosugi *et al* (1991).

In each of the preceding embodiments of the present invention, a Cdc25 substrate or modified Cdc25 substrate protein, in particular the Cdc2a or Cdc2aA14F15 protein, is expressed under the operable control of a plant-expressible promoter sequence. As will be known to those skilled in the art, this is generally achieved by introducing a gene construct or vector into plant cells by transformation or transfection means. The nucleic acid molecule or a gene construct comprising same may be introduced into a cell using any known method for the transfection or transformation of said cell. Wherein a cell is transformed by the gene construct of the invention, a whole organism may be regenerated from a single transformed cell, using methods known to those skilled in the art.

By "transfect" is meant that the gene construct or vector or an active fragment thereof comprising the gene encoding the Cdc25 substrate or modified Cdc25 substrate, in particular, the gene encoding a Cdc2 protein or a homologue, analogue or derivative thereof, and preferably a gene encoding Cdc2aA14F15, operably under the control of the plant-expressible promoter sequence, is introduced into said cell without integration into the cell's genome.

By "transform" is meant that the gene construct or vector or an active fragment thereof comprising the gene encoding the Cdc25 substrate or modified Cdc25 substrate, in

particular, the gene encoding a Cdc2 protein, or a homologue, analogue or derivative thereof, and preferably a gene encoding Cdc2aA14F15, operably under the control of the plant-expressible promoter sequence, is stably integrated into the genome of the cell.

Accordingly, in a further preferred embodiment, the present invention provides a method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising

(i) introducing to a plant cell, tissue or organ a gene construct or vector comprising a nucleotide sequence that encodes a Cdc25 substrate or modified Cdc25 substrate, such as a Cdc2 protein or modified Cdc2 protein or a homologue, analogue, or derivative thereof, in particular Cdc2a or Cdc2aA14F15 protein, operably in connection with a plant-expressible promoter sequence selected from the list comprising strong constitutive promoter sequences; cell-specific promoter sequences, tissue-specific promoter sequences, cell cycle-specific gene promoter sequences, inducible promoter sequences and organ-specific promoter sequences; and

(ii) expressing said protein in one or more of said cells, tissues or organs of the plant.

In an alternative embodiment, the inventive method comprises regenerating a whole plant from the transformed cell.

Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or

in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985), amongst others.

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the gene construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion).

Preferably, the transformed plants are produced by a method that does not require the application of exogenous cytokinin and/or gibberellin during the tissue culture phase, such as, for example, an *in planta* transformation method. In a particularly preferred embodiment, plants are transformed by an *in planta* method using *Agrobacterium tumefaciens* such as that described by Bechtold *et al.*, (1993) or Clough *et al* (1998), wherein *A. tumefaciens* is applied to the outside of the developing flower bud and the binary vector DNA is then introduced to the developing microspore and/or macrospore and/or the developing seed, so as to produce a transformed seed without the exogenous application of cytokinin and/or gibberellin. Those skilled in the art will be aware that the selection of tissue for use in such a procedure may vary, however it is preferable generally to use plant material at the zygote formation stage for *in planta* transformation procedures.

Without being bound by any theory or mode of action, the inventors have discovered

that cytokinin modifies the phosphorylation of a Cdc25 substrate, in particular a Cdc2 protein and, as a consequence, the transformation and/or regeneration of plants in the absence of cytokinin facilitates the recovery of plants which express said Cdc25 substrate and functional homologues, analogues and derivatives thereof at a sufficiently high level to show significant differences from wild-type non-transformed plants. Accordingly, the transformation of plants using gene constructs comprising nucleotide sequences encoding substrates or modified substrates of Cdc25, in particular a Cdc2 protein, such as, but not limited to, Cdc2a or modified Cdc2a, and, in particular Cdc2aA14F15, placed operably under the control of strong, constitutive promoter sequences, are particularly preferred for the purposes of modifying plant morphological, biochemical and physiological properties or characteristics in accordance with the present invention.

A further aspect of the present invention clearly provides the gene constructs and vectors designed to facilitate the introduction and/or expression and/or maintenance of the cell cycle control protein-encoding sequence and plant-expressible promoter into a plant cell, tissue or organ.

In addition to the cell cycle control protein-encoding sequence and plant-expressible promoter sequence, the gene construct of the present invention may further comprise one or more terminator sequences.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3′-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3′-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays zein* gene terminator sequence, the *rbcs-1A* gene terminator, and the *rbcs-3A* gene terminator sequences, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The gene constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell.

Preferred origins of replication include, but are not limited to, the *f1*-ori and *col*E1 origins of replication.

The gene construct may further comprise a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance

(Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

A further aspect of the invention clearly extends to a plant cell, tissue, organ or whole plant that has been transformed or transfected with an isolated nucleic acid molecule that comprises a nucleotide sequence which encodes a cell cycle control protein, wherein the expression of said nucleotide sequence is placed operably under the control of a plant-expressible cell-specific promoter sequence, plant-expressible tissue-specific promoter sequence, plant-expressible cell cycle specific gene promoter sequence, or plant-expressible organ-specific promoter sequence or inducible promoter.

Alternatively, the invention extends to a plant cell, tissue, organ or whole plant that has been transformed or transfected with an isolated nucleic acid molecule that comprises a nucleotide sequence which encodes the cell cycle control protein, placed operably under the control of a plant-expressible constitutive promoter sequence, such that said plant-expressible constitutive promoter sequence and said nucleotide sequence encoding a cell cycle control protein are integrated into a transposable genetic element.

The present invention is applicable to any plant, in particular a monocotyledonous plants and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica,*

Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, brussel sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugarbeet, sugar cane, sunflower, tomato, squash, and tea, amongst others, or the seeds of any

plant specifically named above or a tissue, cell or organ culture of any of the above species.

Accordingly, the present invention clearly extends to any plant produced by the inventive method described herein, and any and all plant parts and propagules thereof. The present invention extends further to encompass the progeny derived from a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by the inventive method, the only requirement being that said progeny exhibits the same genotypic and/or phenotypic characteristic(s) as that (those) characteristic(s) that has (have) been produced in the parent by the performance of the inventive method.

By "genotypic characteristic" is meant the composition of the genome and, more particularly, the introduced gene encoding the cell cycle control protein.

By "phenotypic characteristic" is meant one or more plant morphological characteristics and/or plant biochemical characteristics and/or plant physiological characteristics that are produced by ectopic expression of a cell cycle control protein in a plant.

Preferably, the plant is produced according to the inventive method is transfected or transformed with a genetic sequence, or amenable to the introduction of a protein, by any art-recognised means, such as microprojectile bombardment, microinjection, *Agrobacterium*-mediated transformation (including *in planta* transformation), protoplast fusion, or electroporation, amongst others.

The present invention is further described with reference to the following non-limiting Examples and to the drawings.

EXAMPLE 1

Modification of growth, vigour, flowering time and seed set by ectopic expression of Cdc2a under control of the CaMV 35S promoter in plants

Transformation procedures

A binary plasmid vector containing the *A. thaliana Cdc2a* gene (hereinafter "*Cdc2aAt*") under control of the CaMV 35S promoter, and a kanamycin resistance gene selectable marker was introduced into the bacterium *Agrobacterium tumefaciens* by triparental mating. The transformed *A. tumefaciens* was then introduced into wounded roots from which genetically-transformed kanamycin resistant callus was derived, and shoot and root formation was stimulated by adjustment of auxin to cytokinin ratio during culture to regenerate transgenic plants, essentially as described by Hemerly *et al.* (1995).

Transgenic plants were derived from separate plant populations and are therefore the products of independent transformation events. Individuals having the introduced gene construct at a single point of insertion were selected according to their segregation ratio for the kanamycin-resistance phenotype when selfed. In particular, those plants giving a 3:1 (resistance: sensitivity) segregation ratio were selected. The progeny of these homozygous plants were analysed further for their growth and vigour characteristics compared to otherwise isogenic non-transformed plants.

Determination of plant growth and vigour and total biomass

Plants were grown in containers that are permeable to atmospheric gases, on Murashige and Skoog (1962) mineral salts medium containing 0.75 %(w/v) agar, and without added sugar, at 25°C, and a moderate light intensity of about 80 µmol $m^{-2}sec^{-1}$.

After 11 days of growth under these conditions, transformed plants were significantly larger in size than otherwise isogenic non-transformed plants (Figure 1). As shown in Table 2, these transformed plants also exhibited approximately twice the biomass of the non-transformed plants, as determined by the fresh weight of the plants. The

increased biomass was significant using a standard paired t-test (p=0.001) as described essentially by Snedicore and Cochran (1967).

Table 2

Stimulation of growth in *A. thaliana* by ectopic expression of the *Cdc2a* gene under control of the CaMV 35S promoter

plant type	average weight per plant (mg)	fold stimulation
Wild Type *A. thaliana* C24	1.95	1.00
Cdc2a homozygous transgenic line	4.01	2.06

Increase in Cdc2a protein in the transformed plants

The amount of Cdc2a protein was determined by western blotting using two antibodies:

(i) anti-(PSTAIR) antibodies, which are specific for all CDKs having the PSTAIR amino acid sequence motif, including Cdc2a, modified Cdc2a and any other endogenous CDK present in the cell; and

(ii) anti-(Cdc2a carboxyl terminal peptide) antibodies, which are specific for wild-type Cdc2a and is able to discriminate between endogenous (wild-type) Cdc2a and modified Cdc2a having an altered C-terminus.

Data presented in Figure 2 and Table 3 further indicate that the level of Cdc2a protein in the transgenic lines is significantly increased relative to the steady-state level detected in non-transformed plants. Accordingly, there is a positive correlation between the increased Cdc2a protein and the increased biomass and increased growth and vigour of the transgenic lines.

Table 3

C24 WT and Cdc2a transgenics: amount of Cdc2a protein

Genotype	Amount of Cdc2a protein (relative units)	PSTAIR protein (relative units)
WT C24	1	1
Cdc2a transgenic	11.1	5.8

Flowering times and seed set

We also determined the times to flowering and seed set for the transformed and non-transformed plants.

Flowering time for both transformed and non-transformed plants was determined as the number of days from germination to the opening of the first flower on a plant and reflexing of the petals of said flower, following growth at 21°C in a photoperiod comprising 18 hour light (80 µmol $m^{-2}sec^{-1}$) and 6 hour dark.

Time to seed set for both transformed and non-transformed plants was determined as the number of days from germination to the attainment of a first silique of length greater than 7 mm. As will be known to those skilled in the art, silique elongation in *A. thaliana* is an indicator of seed development and formation in that plant.

As shown in Table 4, there was an approximately 15-20% reduction in both flowering time and time to seed set in homozygous, transformed lines of plants, compared to otherwise isogenic non-transformed *A. thaliana* Columbia plants. This corresponds to a reduction in approximately 8 days in the time to flowering and seed set in these plants.

Table 4

C24 WT and Cdc2a transgenics: Flowering and fruiting timings

Genotype	Ave. days to first flower stage	Ave. days advancement in flowering (% of WT)	Ave. days to first silique formation	Ave. days advancement in first silique formation (% of WT)
WT C24	41.3	-	44.3	-
Cdc2a transgenic	33.2	8.1 d (19%)	36.2	8.1 d (18%)

Determination of shoot biomass, branching, fruit (silique) number and seed size

We also determined the total air-dry weight, number of flowering branches, number of fruits (siliques) per plant and the average seed weight for the transformed and non-transformed plants.

As shown in Table 5, there is a 14% increase in air-dry weight of the shoot biomass of transformed plants, a 66% increase in lateral shoot formation, and a 47% increase in the number of fruits of plants. There was no significant increase in individual seed size, as determined by seed weight.

Table 5

C24 WT and Cdc2a transgenics: Shoot biomass, branching, fruit (silique) number and seed size

Genotype	Air-dry weight of shoot (g)	Number of flowering branches per plant	Number of fruits per plant	Ave. weight per seed (µg)
WT C24	0.44	12	99	22.7
Cdc2a transgenic	0.50	20	146	22.04

EXAMPLE 2

Modification of growth, vigour, flowering time and seed set by ectopic expression of modified Cdc2a (Cdc2aA14F15) under control of the CaMV 35S promoter in plants

Transformation procedures

A binary plasmid vector containing a modified *A. thaliana Cdc2a* gene which expresses a non-phosphorylateable Cdc2a protein (hereinafter "*Cdc2aA14F15*"; Hemerly *et al.*, 1995) under control of the CaMV 35S promoter, and a kanamycin resistance gene selectable marker was introduced into *Agrobacterium tumefaciens* by triparental mating. The transformed *A. tumefaciens* was then introduced into the developing flower bud of *A. thaliana* plants and the seeds developing therefrom were germinated on mineral salts medium supplemented with thiamine and myoinositol (Murashige and Skoog 1962), and containing kanamycin (50 µg/ml) and 30 mM sucrose. Selection was made at an early stage of growth purely on the basis of kanamycin resistance.

Individuals having the introduced gene construct at a single point of insertion were selected according to their segregation ratio for the kanamycin-resistance phenotype when selfed. In particular, those plants giving a 3:1 (resistance: sensitivity) segregation ratio were selected. The progeny of these homozygous plants were

analysed further for their growth and vigour characteristics compared to otherwise isogenic non-transformed plants.

To confirm the presence of the introduced gene construct in the transformed lines, PCR was performed using amplification primers that specifically detect the presence of either the wild-type *Cdc2a* gene or alternatively, the presence of the mutant *Cdc2aA14F15* gene. In particular, the presence of the wild-type *Cdc2a* gene was confirmed using the primer pair:

Primer 1: (WT 5'): 5′-GTTGAGAAGATTGGTGAAGGAACTTA-3′; and

Primer 3:(common 3'): 5′-GTTGAGAAGATTGGTGAAGGAGCTTT-3′.

The presence of the mutant *Cdc2aA14F15* gene was detected using the primer pair:

Primer 2:(Cdc2aA14F15 5'): 5′-CCAAGATCCTTGAAGTATTCATGCTCC-3′; and

Primer 3:(common 3'): 5′-GTTGAGAAGATTGGTGAAGGAGCTTT-3′.

Reactions were commenced by the addition of DNA, or alternatively, by the addition of water for negative controls samples. Amplification reactions were performed in a total volume of 100 µl comprising 1 µl plant DNA (0.15 µg/µl); 74 µl sterile H_2O; 10 µl Taq polymerase buffer; 10 µl 50% (v/v) glycerol; 2.5 µl deoxynucleoside triphosphate mix (2.5 mM each of dATP, dTTP, dCTP, dGTP); 1 µl forward primer (25 µM stock); 1 µl reverse primer (25 µM stock); and 0.5 µl Taq polymerase (2.5 U). Reaction conditions were as follows:

(i) An initial incubation for 4 min at 95°C; and

(ii) 30 cycles each consisting of: (a) 95°C for 15 seconds;

(b) 50°C for 20 seconds; and

(c) 72°C for 30 seconds.

In this PCR test, the product size expected to be amplified is 856 bp in length. Data presented in Figure 3 indicate that a band of the expected size was present in one

putatively transformed sample using primers that specifically detect the mutant *Cdc2aA14F15* gene (see lane 7), but not using primers that detect the wild-type *Cdc2a* gene (Lane 6). In the control samples, a band of the same size was only detected in non-transformed plants using primers that detect the wild-type *Cdc2a* gene (Lane 3). No amplification products were detected in control samples having no added DNA.

Determination of plant growth and vigour and total biomass

Plants were grown in containers that are permeable to atmospheric gases, on Murashige and Skoog (1962) mineral salts medium containing 0.75 %(w/v) agar, and without added sugar, at 25°C, and a moderate light intensity of about 80 µmol $m^{-2}sec^{-1}$.

After 11 days of growth under these conditions, transformed plants were significantly larger in size than otherwise isogenic non-transformed plants (Figure 4).

As shown in Table 6, these transformed plants also exhibited approximately 5- to 9-fold the biomass of the non-transformed plants, as determined by the fresh weight of the plants. The increased biomass was significant using a standard paired t-test (p=0.001) as described essentially by Snedicore and Cochran (1967).

Table 6

Stimulation of growth in *A. thaliana* by ectopic expression of the *Cdc2aA14F15* gene under control of the CaMV 35S promoter

Plant type	Average weight per plant (mg)	Fold stimulation
wild type Columbia	2.05	
1B1C1"	11.6	5.7
1B1D4	18.0	8.8
1C2C5	11.2	5.5

Chlorophyll content

To determine whether the increased size was related to an increased cytoplasm in the transformed lines, we measured total chlorophyll in these plants, by extracting shoot tissue in the dark, for 18 h at 20°C, using N,N'-dimethylformamide. Chlorophyll a (*Chl a*) and chlorophyll b (*Chl b*) levels were estimated by absorbance at 663.8 nm and 646.8 nm, respectively, as described by Porra *et al.* (1989).

Data presented in Table 7 indicate that the increased growth and vigour and total biomass of the transformed plant lines is commensurate with an increase in total chlorophyll content, and the contents of both chlorophyll a and chlorophyll b. In particular, the transformed plants showed an increase in chlorophyll content, expressed as a proportion of fresh weight, compared with non-transformed plants, indicating that the increased growth is a real increase in plant material and not due to mere dilution of the cytoplasm.

Furthermore, there was an unexpected increase of 13% to 53% in chlorophyll content per fresh weight induced in the transgenic plants. Possibly, this increased chlorophyll content explains how the increase rate of cell production is supported in the transgenic lines, such as D4, that grow faster than other lines.

Table 7

Chlorophyll content (μg per gram fresh weight) of shoot tissue in *A. thaliana* that ectopically express the *Cdc2aA14F15* gene

plant type	*Chl a*	*Chl b*	fold increase in *chl a*	fold increase in *chl b*
Wild type Columbia	535	155		
1B1C1	604	179	1.13	1.15
1B1D4	820	240	1.53	1.54
1C2C5	748	213	1.39	1.37

Nuclear DNA content

Nuclei of the transformed line 1B1D4, and of non-transformed plants, were extracted by slicing tissue into nuclear extraction buffer. Nuclei were then stained using DAPI (4,6-diamidino-2-phenylindole) and scanned by flow cytometry, with UV excitation at 395 nm, to determine nuclear DNA content.

Data presented in Table 8 indicate that there was no endoreduplication occurring in the transformed plants which might have produced an increased cell size and thereby account for the observed increase in growth and total biomass of the transformed plants. Data presented in Figure 6, which indicate the absence of any increased cell size in the transformed lines, confirm these data.

Table 8

Nuclear DNA content of *A. thaliana* that ectopically express the *Cdc2aA14F15* gene under control of the CaMV 35S promoter

nuclear DNA content (multiple of haploid C value)	Wild type	transgenic line 1B1D4
2C	39.8%	45.9%
4C	37.2%	30.2%
8C	9.6%	9.3%
16C	3.8%	1.7%

Increase in Cdc2a protein in the transformed plants

The level of Cdc2a protein in the transformed plants was determined using western blotting as described in Example 1.

Data presented in Figure 5 and Table 9 indicate that the level of Cdc2a protein in the

transgenic lines is significantly increased relative to the steady-state level detected in non-transformed plants. The transgenic line 5A2 8C does not show any elevation of Cdc2a protein level and may have integrated only the Kanamycin resistance gene with the modified Cdc2a gene.

In summary, there is a positive correlation between the increased Cdc2a protein and the increased biomass and increased growth and vigour of the transgenic lines.

The increase in signal from the transgenic plants that was indicated by the anti-(Cdc2a carboxyl terminal) antibody indicates that the PSTAIR antibody detects additional species of endogenous CDK (i.e. other PSTAIR containing proteins). This means that Cdc2a is not the only PSTAIR containing CDK in *A. thaliana,* and that the endogenous level of all PSTAIR CDKs combined is higher than the endogenous level of Cdc2a.

The Cdc2a protein is increased in plants for several reasons. First, the number of expressible *Cdc2a* genes in the transformed plants is increased. Second, the size of the meristem in the transformed plants is increased and, as a consequence, a higher proportion of cells in the transformed plants which express Cdc2a at a high level is increased. Third, the CaMV 35S promoter which regulates expression of both the introduced *Cdc2a* gene and the modified *Cdc2aA14F15* gene is stronger than the endogenous *Cdc2a* gene promoter.

Determination of cell size

Cell size for transformed and non-transformed plants was determined by examining the lower leaf epidermis of unfixed turgid leaves using a Zeiss Axiovert 35M at X40 magnification, with DIC optics and Metamorph 4.0 software.

Results are presented in Figure 6. In each image presented in Figure 6, 1 cm corresponds to 25 μm of leaf.

Data presented in Figure 6 indicate that there is no difference in cell size between transformed and non-transformed lines. Accordingly, we conclude that the increased size and biomass of plants transformed with either the mutant *Cdc2aA14F15* gene or the wild-type *Cdc2a* gene is due to an increase in cell number. Such an increase in cell number may be the result of more rapid cell cycling and/or an increase in the number of cells dividing (i.e. larger meristematic centres).

Table 9

C24 WT and Cdc2aA14F15 transgenics: amount of Cdc2a protein

Genotype	Amount of Cdc2a protein (relative units)	PSTAIR protein (relative units)
WT ANU Columbia	1	1
1B1 B4	11	5.1
1B1 C1	37	8.3
1B1 D4	47	9.9
1C2 C5	1.8	1.6
4A1 L2	17	5.9
4A2 B2	5.8	3.2
5A2 8C	0.9	1.1
5B2 G4	22	5.7
6B1 B4	2	1.5
6B2 F5	not assayed	not assayed

Flowering times and seed set

We also determined the times to flowering and seed set for the transformed and non-transformed plants.

Flowering time for both transformed and non-transformed plants was determined as the number of days from germination to the opening of the first flower on a plant and reflexing of the petals of said flower, following growth at 21°C in a photoperiod comprising 18 hour light (80 µmol $m^{-2}sec^{-1}$) and 6 hour dark.

Time to fruit (silique) formation for both transformed and non-transformed plants was determined as the number of days from germination to the attainment of a first silique of length greater than 7 mm. As will be known to those skilled in the art, silique elongation in *A. thaliana* is an indicator of seed development and formation in that plant.

Early flowering time was observed, albeit not for all transformed lines tested, although increase in yield is invariable in plants expressing moderately elevated levels of Cdc2a protein. In those plants in which increased growth was not fully reflected in increased branching, the flowering spike developed more rapidly than in wild type. This occurred in transgenic lines with moderately raised levels of modified Cdc2a between 3 and 6 fold higher than the control.

As shown in Table 10, flowering time is reduced by up to about 10% in the homozygous, transformed lines of plants, compared to otherwise isogenic non-transformed *A. thaliana* Columbia plants. This corresponds to a reduction in at least up to 3-4 days in the time to flowering for these transformed plants. Similar results were obtained for the time to seed set in the transformed plant lines. However, these data are qualified by the fact the, at the time these measurements were taken, not all of the control wild-type non-transgenic plants had flowered. As a consequence, the real reduction in both flowering time and seed set for the transformed lines is greater than that indicated in Table 10.

Moreover, the transformed lines that grew the fastest in the first 11 days from germination are generally not earlier-flowering than those lines that grew more slowly.

In particular, the earliest-to-flower transformed lines were about twice the size of the control plants at 11 days, whilst the larger plants flowered more slowly. Possibly, in the larger plants, increased growth potential had been more directed to producing lateral branches than to early flower production.

Table 10

Columbia WT and Cdc2aA14F15 transgenics: Flowering and fruiting timings

genotype	Ave. days to first flower	Ave. days advancement in flowering (% relative to wild-type)	Ave. days to first silique formation	Ave. days advancement in silique formation (% relative to wild-type)
WT ANU Columbia	31.7	-	33.9	-
1B1 B4	32.1	none	35	none
1B1 C1	30.1	1.6 d (5.2%)	34	none
1B1 D4	32.1	none	34	none
1C2C5	32.1	none	35	none
4A1 L2	29.6	**2.1 d (6.6%)**	32.3	**1.6 d (5.1%)**
4A2 B2	28.5	**3.2 d (10.2%)**	30.3	**3.6 d (10.6%)**
5A2 8C	31.1	0.6 d (11.9%)	34.1	none
5B2 G4	28.8	**2.8 d (9.6%)**	31.1	**2.8 d (8.7%)**
6B1 B4	30.8	0.9 d (3.1%)	32	1.9 d (5.6%)
6B2 F5	28.4	**3.3 d (10.6%)**	29.7	**3.4 d (10.7%)**

Determination of shoot biomass, branching, fruit (silique) number and seed size

We also determined the total air-dry weight, number of flowering branches, number of

fruits (siliques) per plant and the average seed weight for the transformed and non-transformed plants.

As shown in Table 11, there is a 14% increase in air-dry weight of the shoot biomass of transformed plants, a 66% increase in lateral shoot formation, and a 40% increase in the number of fruits of plants. There was no significant increase in individual seed size, as determined by seed weight. These percentage changes in shoot biomass and branching are similar to those observed when the wild-type Cdc2a protein was ectopically-expressed in plants (see Table 5).

Table 11

C24 WT and Cdc2aA14F15 transgenics: Shoot biomass, branching, fruit (silique) number and seed size

Genotype	Air-dry weight of shoot (g)	Number of flowering branches per plant	Number of fruits per plant	Ave. weight per seed (µg)
WT C24	0.49	15	92	18.5
Cdc2aA14F15 transgenic line 1B1B4	0.56	25	127	19.37

EXAMPLE 3

Expression of Cdc2a under the control of the patatin gene promoter increases tuber size and number in potato plants

The *A. thaliana* Cdc2a coding sequence is cloned between the promoter of a class I patatin gene (Liu *et al.*,1991) and the transcription termination signals of the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*. Preferentially, the B repeat region and the distal region of the A repeat of the patatin promoter is used, without the proximal region of the A repeat. The proximal region of the A repeat of the patatin

promoter confers sucrose-responsiveness in various tissues, which is not a desirable characteristic for our purposes (Grierson *et al.,* 1994). This construct is placed in a binary vector, mobilized to *Agrobacterium tumefaciens*, and the introduced into potato plants.

The Cdc2a protein is expressed under the control of the Class I patatin promoter when the first stolon starts to tuberize, consistent with the expression pattern for the patatin gene (Liu *et al.,* 1991). At this stage, expression is associated with both internal and external phloem. After tuber induction has occurred, promoter activity is found both in tuberized stolons and in non-tuberized stolons. Expression then expands to the entire storage parenchyma, cortex and pith, but remains absent from the periderm.

Because the Class II patatin promoters are expressed in the periderm and as such are complementary to the Class I promoters (Köster-Töpfer *et al.,*; Liu *et al.,* 1991; Nap *et al.,* 1992), it is beneficial to have Cdc2a expression driven by both Class I and Class II promoters within the same plant. Because the Class I patatin promoter is not expressed before the first stolon initiates tuberization, no effects of Class I patatin-Cdc2a transgenes is seen on tuber initiation. However, the Class I patatin promoter drives Cdc2a expression very early after tuber initiation onwards, allowing a maximal impact of Cdc2a activity on organ formation and, as a consequence, on tuber size. The fact that the Class I patatin promoter activity subsequently also appears in non-tuberized stolons implies that the Class I patatin – Cdc2a transgene increases both the size and number of tubers.

EXAMPLE 4

Expression of Cdc2a under the control of the SAUR gene promoter or the *A. rhizogenes rolB* promoter increases lignin in poplar plants

The *A. thaliana* Cdc2a coding sequence is cloned between the promoter of the soybean SAUR gene (Li *et al.,* 1992) and the transcription termination signals of the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*. The SAUR promoter

is inducible by auxins. This chimeric gene construct is introduced between the T-DNA borders of the binary vector pBI121 or similar vector and mobilised into *Agrobacterium tumefaciens*. Poplar is transformed by *Agrobacterium*-mediated transformation using standard procedures.

Transgenic poplar trees containing this construct show increased lignin content, correlated with an increased stem diameter and the higher ratio of vascular tissue to pith and cortex cells.

A similar phenotype in poplar is produced when the Cdc2a expression is driven by the *rolB* promoter of *Agrobacterium rhizogenes* (Nilsson *et al.*, 1997), that is expressed in cambial cells (i.e. the dividing cells of the vascular tissue).

EXAMPLE 5

Expression of Cdc2a under the control of endosperm-specific promoters increases grain size and yield of grain crop plants

The *A. thaliana* Cdc2a coding sequence is placed operably in connection with the endosperm-specific *ltr1* promoter from barley, or a synthetic promoter containing the endosperm box (GCN motif) of the barley *Hor2* gene (Vicente-Carbajosa *et al.*, 1998). In each case, the Cdc2a structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. Cereals, in particular rice, maize, wheat and barley, are transformed using standard procedures, in particular microprojectile bombardment or *Agrobacterium*-mediated transformation systems, with the gene constructs.

The grain size and starch storage capacity of the endosperm of the seeds of transformed plants is increased relative to otherwise isogenic non-transformed plants.

EXAMPLE 6

Expression of Cdc2a under the control of meristem-specific promoters reduces apical dominance in *A. thaliana* and *B. napus* plants

The *A. thaliana* Cdc2a coding sequence is placed operably in connection with the shoot meristem-specific *LEAFY* promoter (Weigel *et al.,* 1992), or the KNOTTED-like *A. thaliana knat1* promoter (Accession number AJ131822), or the KNOTTED-like *Malus domestica kn1* promoter (Accession No. Z71981), or the *A. thaliana CLAVATA1* promoter (Accession number AF049870). In each case, the Cdc2a structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. *A. thaliana* and *Brassica napus* plants are transformed as described by Bechtold *et al.,* 1993.

Transformed plants exhibit cytokinin-like effects at the level of the shoot (and flower) meristem, resulting in reduced apical dominance.

EXAMPLE 7

Expression of Cdc2a under the control of the *cab-6* or *ubi7* promoters reduces leaf necrosis and chlorosis in lettuce plants

The *A. thaliana* Cdc2a coding sequence is placed operably in connection with the leaf-specific *cab-6* gene promoter derived from *Pinus* (Yamamoto *et al.,* 1994) or senescence-specific *ubi7* gene promoter (Garbarino *et al.,* 1995). In each case, the Cdc2a structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. Lettuce is transformed as described by Bechtold *et al.,* 1993.

Leaf deterioration (chlorosis and necrosis) in lettuce, for example as a consequence of post-harvest storage, is delayed in transformed lettuce plants compared to non-transformed control plants.

EXAMPLE 8

Inheritance of the *Cdc2aAt.A14F15* transgene and effect on growth of transgenic *A. thaliana* plants

Populations of 100 seeds derived from the self-fertilisation of hemizygous transgenic lines were equally spaced in Petri dishes permeable to atmospheric gases on Murashige and Skoog (1962) inorganic medium, and were grown photosynthetically as described in Figure 1. The transgenic lines YF-D and YF-E showed a 75% incidence of kanamycin resistance among progeny when the initial hemizygous individual was self-fertilised, indicative of a single point of insertion of the transgenic DNA.

Plants of the transgenic lines YF-D and YF-E were categorised after 11 days of growth, on the basis of whole-seedling fresh weight, and seedling size class (big, mid or small). In each category, 14 individuals were pooled; this being the largest number of individuals that in 99% of cases would be present in a total population of 100 individuals in which a single transgene insertion was segregating.

Plants were also subjected to western blotting as described for Figure 5, to determine Cdc2a protein expression level, and to confirm the presence of the functional transgene. As a control to verify equal protein loading per sample, for accurate quantitation, proteins were separated using SDS/PAGE transferred to PVDF membranes and stained to visualise the transferred proteins, using 0.1% (w/v) polyvinylpyrrolidone-40 followed by immersion in 0.75 mM ferrozine, 20 mM $FeCl_2$, 2% (v/v) glacial acetic, followed by immersion in 100 mM ferrocyanide, 60 mM $FeCl_2$, 100 mM Na-Acetate, buffered at pH4 with glacial acetic acid. After recording the stained image, the membrane was de-stained by immersion in 100 mM Tris, 20 mM EDTA, and adjusted to pH10 with KOH, then blocked with BSA and probed with antibody as described for Figure 5. All plants tested were grown, extracted, electrophoresed on the same gel, and transferred simultaneously to the sane PVDF membrane for antibody probing.

Figure 7 shows the inheritance of the *Cdc2aAt.A14F15* transgene and enhanced growth effects seen in *A. thaliana* seedlings from self-fertilisation of initial transgenic isolate line YF-D (panels A, B) and a different transgenic isolate line YF-E (Panels C, D). Panels (A) and (C) show a Western blot probed with antibody specific for the *cdc2a.At* gene product (the 34 kDa Cdc2a protein is indicated by the arrows), to detect the expression of the transgene (lanes 1-3), relative to the amount of protein detected in a non- transgenic isogenic plant (lane 4).

Data presented in Figure 7 show increased levels of the 34 kDa *A. thaliana* Cdc2aY14F15 polypeptide in both transgenic lines compared to the non-transformed lines, and that expression of this polypeptide is correlated with plant size.

As shown in Table 12, the fresh weight of the plants, measured as the weight of a whole seedling including root and shoot, and the size of the plant, is positively correlated with the amount of Cdc2a protein expressed, as detected in Western blots.

The capacity of the *Cdc2aAt.A14F15* transgene to be segregated out of the slower-growing plants further indicates a single point of insertion of the introduced DNA and confirms that the presence and expression of the transgene induces faster growth of plants.

TABLE 12

Fresh weight and size of transgenic plants expressing Cdc2aAtA14F15 is correlated with the amount of Cdc2aAtA14F15 protein expressed in plants

LINE YF-D				
Plant phenotype	**big**	**mid**	**small**	**WT**
Fresh weight (mg av. at 11 days growth)	9	8	4	5
Amount Cdc2a protein	2.3	1.7	1	1

LINE YF-E				
Plant phenotype	**big**	**mid**	**small**	**WT**
Fresh weight (mg av. at 11 days growth)	8	78	40	5
Amount Cdc2a protein	2.0	2.1	1.1	1

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WE CLAIM:

1. A method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a Cdc25 substrate or modified Cdc25 substrate operably under the control of a promoter sequence that is operable in a plant or a cell, tissue or organ thereof.

2. The method according to claim 1, wherein the Cdc25 substrate is a Cdc2 protein or a modified Cdc2 protein or a homologue, analogue or derivative thereof.

3. The method according to claim 2, wherein the Cdc2 protein is a Cdc2a protein or a modified Cdc2a protein or a homologue, analogue or derivative thereof.

4. The method according to claim 2, wherein the Cdc2 protein or modified Cdc2 protein is a Cdc2 protein of fission yeast.

5. The method according to claim 2, wherein the Cdc2 protein or modified Cdc2 protein is a Cdc2 protein of plants.

6. The method according to claim 5, wherein the Cdc2 protein or modified Cdc2 protein is a Cdc2a protein of plants or modified Cdc2a protein of plants.

7. The method according to claim 6, wherein the plant is a dicotyledonous plant.

8. The method according to claim 7, wherein the dicotyledonous plant is *A. thaliana*.

9. The method according to claim 1, wherein the modified Cdc25 substrate contains one or more modified amino acid residues that produce a non-phosphorylateable protein or a dephosphorylated protein.

10. The method according to claim 9, wherein the modified Cdc25 substrate comprises a threonine residue at amino acid position-14 and/or a tyrosine residue at amino acid position-15.

11. The method according to claim 10, wherein the modified Cdc25 substrate is Cdc2aA14F15.

12. The method according to claim 1, wherein the promoter sequence is a constitutive promoter sequence.

13. The method according to claim 12, wherein the constitutive promoter sequence is the CaMV 35S promoter sequence or the *gos2* gene promoter sequence.

14. The method according to claim 1, wherein the promoter sequence is an inducible promoter sequence.

15. The method according to claim 14, wherein the inducible promoter sequence is a dexamethasone-inducible promoter sequence.

16. The method according to claim 15, wherein the dexamethasone-inducible promoter sequence comprises one or more modified glucocorticoid response elements (GREs).

17. The method according to claim 1, wherein the modified plant morphological

and/or biochemical and/or physiological characteristic comprises enhanced growth and/or enhanced vigour of the plant.

18. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises increased total biomass of the plant.

19. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises increased cell number.

20. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises reduced flowering time.

21. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises increased inflorescence formation.

22. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises reduced time to seed set.

23. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic produced by ectopic expression of the Cdc25 substrate or modified Cdc25 substrate is selected from the group consisting of: (i) enhanced seed set; (ii) enhanced seed size; (iii) enhanced grain yield; and (iv) enhanced endoreduplication in the seed of the plant.

24. The method according to claim 23, wherein ectopic expression of the Cdc25 substrate or modified Cdc25 substrate is regulated by a constitutive promoter sequence or a promoter sequence that is at least operable in the seed of a plant or a cell, tissue or organ of said seed.

25. The method according to claim 24, wherein the promoter sequence is selected from the group consisting of: (i) a barley *Amy32b* gene promoter sequence; (ii) a Cathepsin β-like gene promoter sequence; (iii) a wheat ADP-glucose pyrophosphorylase gene promoter sequence; (iv) a maize zein gene promoter sequence; (v) a rice glutelin gene promoter sequence; (vi) a legumin gene promoter sequence; (vii) a *napA* gene promoter sequence; (viii) a Brazil Nut albumin gene promoter sequence; (ix) a pea vicilin gene promoter sequence; (x) a sunflower oleosin gene promoter sequence; (xi) a barley *blz2* gene promoter sequence; (xii) a barley *ltr1* gene promoter sequence; and (xiii) a barley *Hor2* gene promoter sequence.

26. The method according to claim 24, wherein the promoter sequence comprises a rice prolamin *NRP33* promoter sequence.

27. The method according to claim 24, wherein the promoter sequence comprises a synthetic promoter that contains a rice *REB* gene promoter sequence.

28. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises one or more modified cytokinin-mediated characteristics relative to an otherwise isogenic non-transformed plant.

29. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic is selected from the group consisting of: enhanced strength, enhanced stem thickness, enhanced stability, and

enhanced wind-resistance, and wherein the promoter sequence is at least operable in the stem of a plant or a cell, or tissue thereof.

30. The method according to claim 29, wherein the promoter sequence is selected from the group consisting of: (i) a *rbcs-1A* gene promoter sequence; (ii) a *rbcs-3A* gene promoter sequence; (iii) a *AtPRP4* gene promoter sequence; (iv) a *T. bacilliform* virus gene promoter sequence; and (v) a sucrose-binding protein gene promoter sequence.

31. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic is selected from the group consisting of: enhanced tuber formation and enhanced tuber development, and wherein the promoter sequence is at least operable in the tuber of a plant or a cell, or tissue of said tuber.

32. The method according to claim 31, wherein the plant is potato.

33. The method according to claim 31 wherein the promoter sequence is a potato patatin gene promoter sequence.

34. The method according to claim 33, wherein the patatin gene promoter sequence is selected from the group consisting of: (i) a class I patatin gene promoter sequence; and (ii) a class II patatin gene promoter sequence.

35. The method according to claim 34, wherein the class I patatin gene promoter sequence has a reduced number of functional sucrose-responsive elements compared to the naturally-occurring class I patatin gene from which said promoter sequence was derived.

36. The method according to claim 35 wherein the number of functional sucrose-responsive elements is reduced by deletion of a proximal region of the A repeat in said class I patatin gene.

37. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises modified lignin content, and wherein the promoter sequence is at least operable in the cambium or vasculature of a woody plant, or a cell, tissue or organ of said cambium or vasculature.

38. The method according to claim 37, wherein the promoter sequence is selected from the group consisting of: (i) a cinnamoyl alcohol dehydrogenase (CAD) gene promoter sequence; (ii) a laccase gene promoter sequence; (iii) a cellulose synthase gene promoter sequence; (iv) a *parAs* or *parAt* gene promoter sequence; and (v) a xyloglucan endotransglucosylase (XET) gene promoter sequence.

39. The method according to claim 37, wherein the promoter sequence is the auxin-inducible SAUR promoter sequence.

40. The method according to claim 37 wherein the promoter sequence is the *rolB* promoter sequence.

41. The method according to claim 37, wherein the woody plant is selected from the group consisting of: *Eucalyptus spp.; Populus spp.; Quercus spp.; Acer spp.; Juglans spp.; Fagus spp.; Acacia spp.;* and teak.

42. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced bushiness or reduced apical dominance of the plant, and wherein the promoter sequence is at

least operable in the meristem of a plant or a meristem cell.

43. The method according to claim 42 wherein the meristem is a lateral meristem.

44. The method according to claim 42 wherein the meristem is an apical meristem.

45. The method according to claim 42 wherein the promoter sequence comprises a *LEAFY* gene promoter sequence.

46. The method according to claim 42 wherein the promoter sequence comprises a *knat1* gene promoter sequence.

47. The method according to claim 32 wherein the promoter sequence comprises a *kn1* gene promoter sequence.

48. The method according to claim 42 wherein the promoter sequence comprises a *CLAVATA1* gene promoter sequence.

49. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced lateral root formation of the plant, and wherein the regulatable promoter sequence is at least operable in the root of a plant or a cell, tissue or organ of said root.

50. The method according to claim 49, wherein the promoter sequence comprises a tobacco auxin-inducible gene promoter sequence.

51. The method according to claim 1 wherein the modified plant morphological

and/or biochemical and/or physiological characteristic comprises enhanced nitrogen fixing capacity of the plant or a nodule of said plant, and wherein the regulatable promoter sequence is at least operable in the module of a plant or a cell, or tissue of said nodule.

52. The method according to claim 51, wherein the promoter sequence is selected from the group consisting of: (i) a *nif* gene promoter sequence; (ii) a *nifH* gene promoter sequence; (iii) a ENOD gene promoter sequence; (iv) a PEPC gene promoter sequence; (v) a leghaemoglobin gene promoter sequence; and (vi) a hemoglobin gene promoter sequence.

53. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises reduced or delayed chlorosis and/or necrosis of the green leaf tissue of the plant, and wherein the promoter sequence is at least operable in the leaf of a plant or a cell, or tissue of said leaf.

54. The method according to claim 53, wherein the promoter is selected from the group consisting of: (i) a SAM22 gene promoter sequence; (ii) a *rbcs-1A* gene promoter sequence; (iii) a *rbcs-3A* gene promoter sequence; (iv) a *cab-6* gene promoter sequence; and (v) a *ubi7* gene promoter sequence.

55. The method according to claim 1 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions.

56. The method according to claim 55 wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced

endoreplication and/or enhanced endoreduplication.

57. The method according to claim 1, wherein the modified plant morphological and/or biochemical and/or physiological characteristic comprises enhanced cell expansion.

58. The method according to claim 1, wherein the promoter sequence comprises a regulatable cell-specific promoter sequence.

59. The method according to claim 1, wherein the promoter sequence comprises a regulatable tissue-specific promoter sequence.

60. The method according to claim 59, wherein the regulatable tissue-specific promoter sequence is selected from the group consisting of:(i) a phloem-specific promoter sequence; (ii) a cell-wall-specific promoter sequence; (iii) a root cortex-specific promoter sequence; (iv) a root vasculature-specific promoter sequence; (v) a tapetum-specific promoter sequence; and (vi) a meristem-specific promoter sequence.

61. The method according to claim 1, wherein the promoter sequence comprises a regulatable organ-specific promoter sequence.

62. The method according to claim 61, wherein the regulatable organ-specific promoter sequence is selected from the group consisting of: (i) an aleurone-specific promoter sequence; (ii) a flower-specific promoter sequence; (iii) a fruit-specific promoter sequence; (iv) a leaf-specific promoter sequence; (v) a nodule-specific promoter sequence; (vii) a pollen-specific promoter sequence; (viii) an anther-specific promoter sequence; (ix) a root-specific promoter sequence; (x) a seed-specific promoter sequence; (xi) an endosperm-specific promoter sequence; (xii) an embryo-

specific promoter sequence; and (xiii) a stigma-specific promoter sequence.

63. The method according to claim 1, wherein the promoter sequence comprises a regulatable cell cycle-specific promoter sequence.

64. The method according to claim 63 wherein the regulatable cell cycle-specific promoter sequence comprises a cell cycle gene promoter sequence.

65. The method according to claim 1, wherein the nucleotide sequence encoding the Cdc25 substrate or modified Cdc25 substrate protein is expressed by a process comprising introducing a gene construct that comprises said nucleotide sequence operably in connection with a plant-operable promoter sequence into a plant cell and culturing said plant cell under conditions sufficient for transcription and translation to occur.

66. The method according to claim 65, wherein culturing of the plant cell under conditions sufficient for transcription and translation to occur includes organogenesis or embryogenesis.

67. The method according to claim 66 wherein the organogenesis or embryogenesis includes regeneration of the plant cell into a whole plant.

68. A transformed plant produced by the method according to claim 67.

69. The transformed plant according to claim 68, wherein said plant exhibits one or more modified plant morphological and/or biochemical and/or physiological characteristics compared to otherwise isogenic non-transformed plants selected from the group consisting of: (i) enhanced growth and/or enhanced vigour of the plant; (ii)

increased total biomass of the plant; (iii) increased cell number; (iv) reduced flowering time; (v) increased inflorescence formation; (vi) reduced time to seed set (vii) enhanced seed set; (viii) enhanced seed size; (ix) enhanced grain yield; (x) enhanced endoreduplication in the seed of the plant; (xi) enhanced stem strength; (xii) enhanced stem thickness; (xiii) enhanced stem stability; (xiv) enhanced wind-resistance of the stem; (xv) enhanced tuber formation; (xvi) enhanced tuber development; (xvii) increased lignin content; (xviii) enhanced ploidy of the seed; (xix) enhanced endosperm size; (xx) reduced apical dominance; (xxi) increased bushiness; (xxii) enhanced lateral root formation; (xxiii) enhanced rate of lateral root production; (xxiv) enhanced nitrogen-fixing capability; (xxv) enhanced nodulation or nodule size; (xxvi) reduced or delayed leaf chlorosis; (xxvii) reduced or delayed leaf necrosis; (xxviii) partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions; (xxix) enhanced endoreplication and/or enhanced endoreduplication; and (xxx) enhanced cell expansion.

70. A plant part, propagule, or progeny, of the plant according to claim 69, wherein said plant part, propagule or progeny exhibits one or more modified plant morphological and/or biochemical and/or physiological characteristics of said plant as a consequence of the ectopic expression of the Cdc25 substrate or modified Cdc25 substrate protein therein.

71. A gene construct comprising a nucleotide sequence encoding a Cdc25 substrate or modified Cdc25 substrate protein, placed operably in connection with a promoter sequence that is operable in a plant or a cell, tissue or organ of said plant, wherein said promoter sequence is selected from the group consisting of: (i) a strong constitutive promoter sequence; (ii) a patatin gene promoter sequence; (iii) a modified patatin gene promoter sequence having a deletion in a sucrose-responsive element; (iv) an auxin-inducible SAUR gene promoter sequence; (v) a *rolB* gene promoter sequence; (vi) a rice prolamin *NRP33* gene promoter sequence; (vii) a synthetic promoter sequence comprising one or more endosperm box motifs derived of the

barley *Hor2* gene; (viii) a *LEAFY* gene promoter sequence; (ix) a *knat1* gene promoter sequence; (x) a *kn1* gene promoter sequence; (xi) a *CLAVATA1* gene promoter sequence; (xii) a *cab-6* gene promoter sequence; (xiii) a rice REB gene promoter sequence; and (xiv) a *ubi7* gene promoter sequence.

72. The gene construct according to claim 71 wherein the Cdc25 substrate is a Cdc2 protein or a modified Cdc2 protein or a homologue, analogue or derivative thereof.

73. The method according to claim 72, wherein the Cdc2 protein is a Cdc2a protein or a modified Cdc2a protein or a homologue, analogue or derivative thereof.

74. The method according to claim 73, wherein the modified Cdc2a protein is a Cdc2aA14F15 protein or a homologue, analogue or derivative thereof.

75. A transformed plant comprising the gene construct according to claim 71, wherein said plant exhibits one or more modified plant morphological and/or biochemical and/or physiological characteristics compared to otherwise isogenic non-transformed plants selected from the group consisting of:(i) enhanced growth and/or enhanced vigour of the plant; (ii) increased total biomass of the plant; (iii) increased cell number; (iv) reduced flowering time; (v) increased inflorescence formation; (vi) reduced time to seed set (vii) enhanced seed set; (viii) enhanced seed size; (ix) enhanced grain yield; (x) enhanced endoreduplication in the seed of the plant; (xi) enhanced stem strength; (xii) enhanced stem thickness; (xiii) enhanced stem stability; (xiv) enhanced wind-resistance of the stem; (xv) enhanced tuber formation; (xvi) enhanced tuber development; (xvii) increased lignin content; (xviii) enhanced ploidy of the seed; (xix) enhanced endosperm size; (xx) reduced apical dominance; (xxi) increased bushiness; (xxii) enhanced lateral root formation; (xxiii) enhanced rate of lateral root production; (xxiv) enhanced nitrogen-fixing capability; (xxv) enhanced

nodulation or nodule size; (xxvi) reduced or delayed leaf chlorosis; (xxvii) reduced or delayed leaf necrosis; (xxviii) partial or complete inhibition of the arrest of DNA replication in a plant cell under growth-limiting conditions; (xxix) enhanced endoreplication and/or enhanced endoreduplication; and (xxx) enhanced cell expansion.

76. A plant part, propagule, or progeny, of the plant according to claim 75, wherein said plant part, propagule or progeny exhibits one or more of the modified plant morphological and/or biochemical and/or physiological characteristics of said plant as a consequence of the ectopic expression of the Cdc25 substrate or modified Cdc25 substrate protein therein.

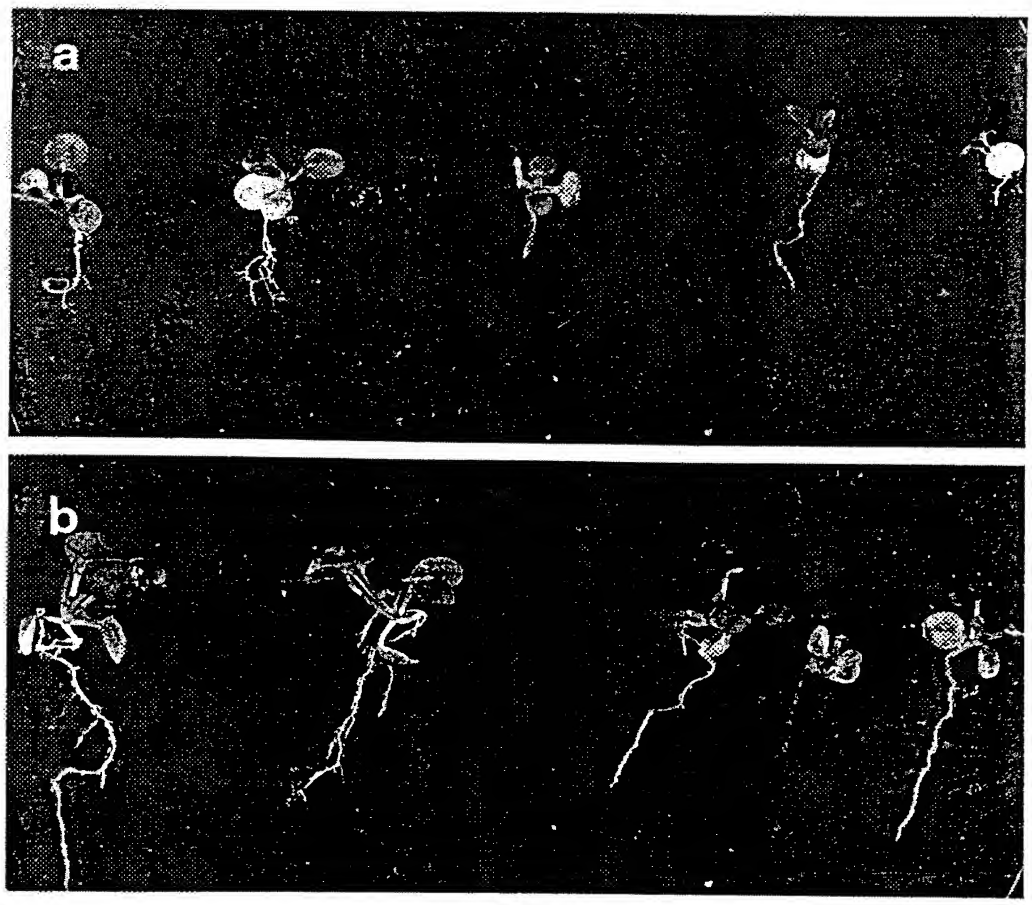
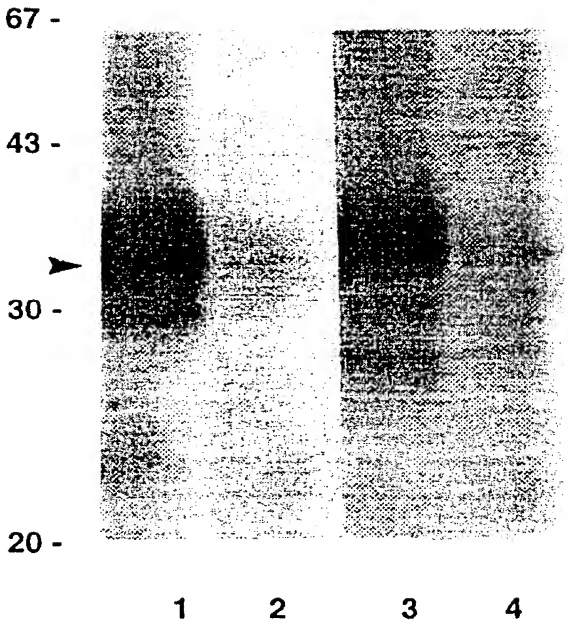
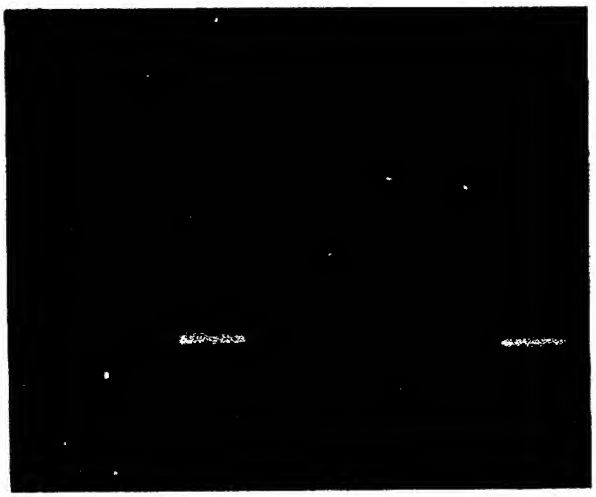


FIGURE 1



Amounts of CDK protein in wild type Arabidopsis line C24 (lanes 2,4), and in seedlings of a transgenic line (lanes 1,3) expressing additional copies of the cdc2aAt gene . Lanes 1,2 Cdc2 family proteins containing PSTAIR peptide (EGVPSTAIREISLLKE). Lanes 3,4 Cdc2a protein. Equal loadings of total extracted proteins from 11 day seedlings of each type were subject to western blot analysis with antibody specific for the PSTAIR peptide (lanes 1,2) or the carboxyterminal sequence of Arabidopsis Cdc2a (lanes 3,4). Ordinate label shows mobility of calibration proteins of indicated molecular ratio (kDa) and arrow indicates 34 kDa Cdc2a protein.

FIGURE 2



lane	1	2	3	4	5	6	7
primers		1+2	1+2	3+2	3+2	1+2	3+2

FIGURE 3

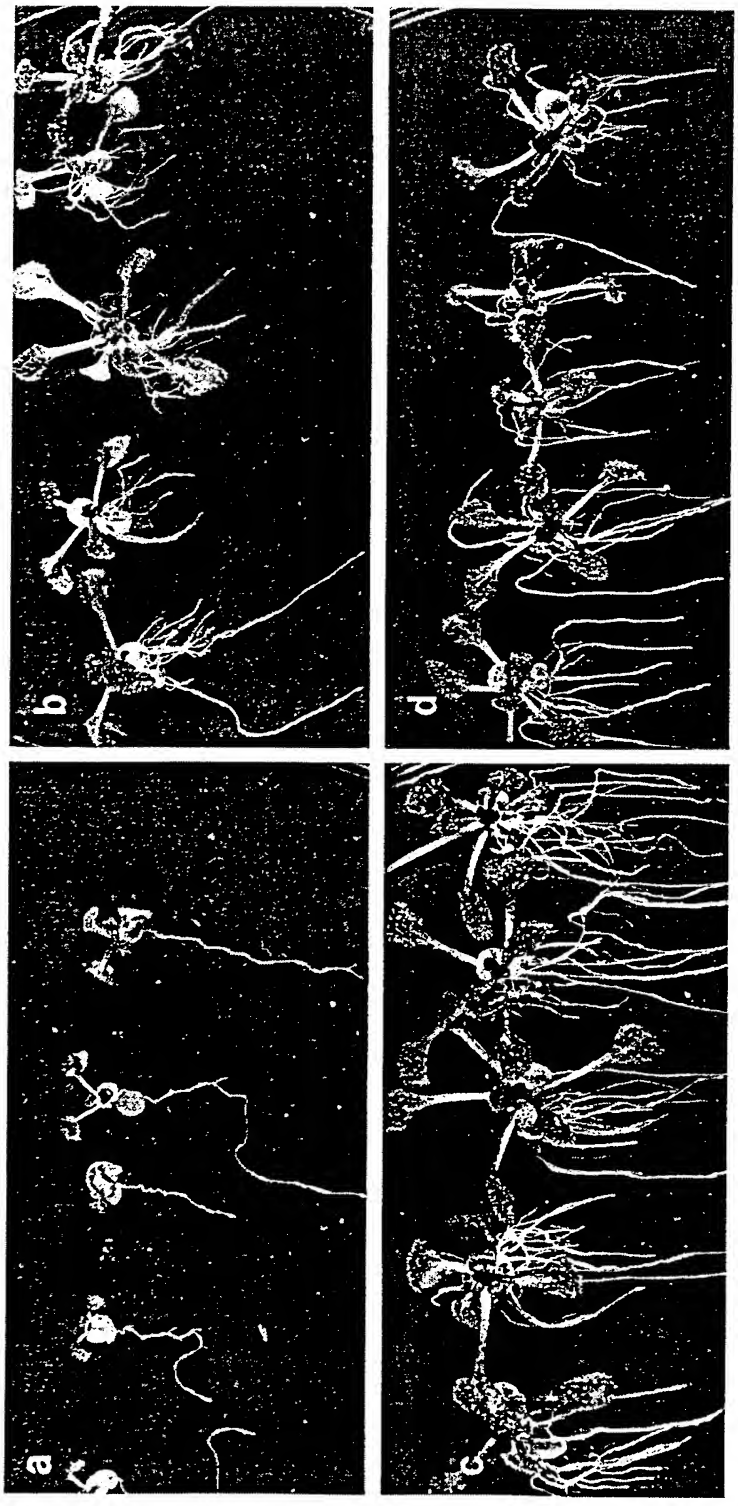
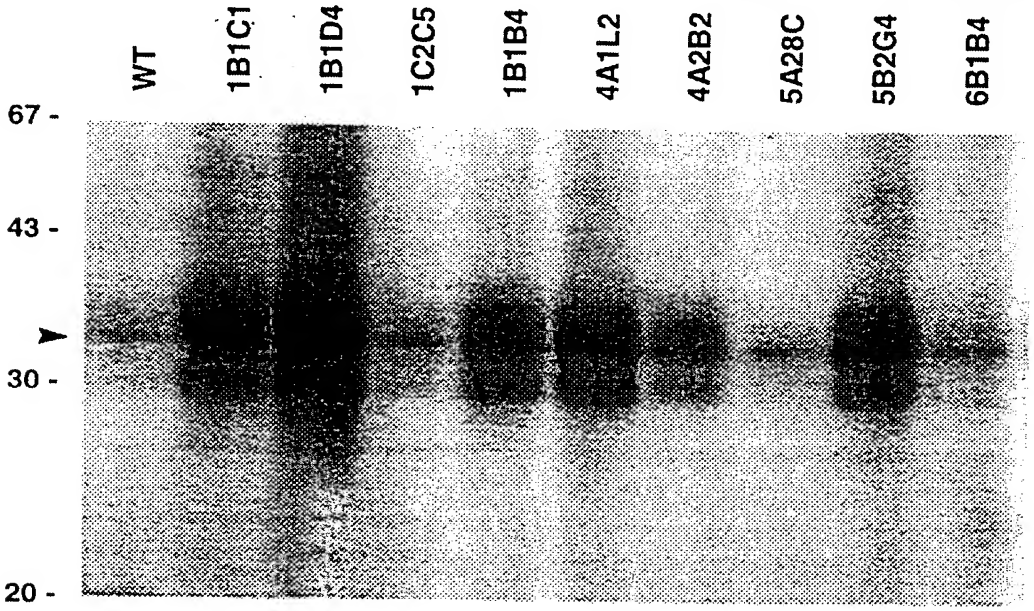


FIGURE 4

Protein levels of CDK containing PSTAIR and modified CDK (Cdc2aT/A14F15) enzyme in wild type and transgenic Arabidopsis



Protein levels of endogenous Cdc2a and modified Cdc2a (Cdc2aT/A14F15) enzyme in wild type and transgenic Arabidopsis

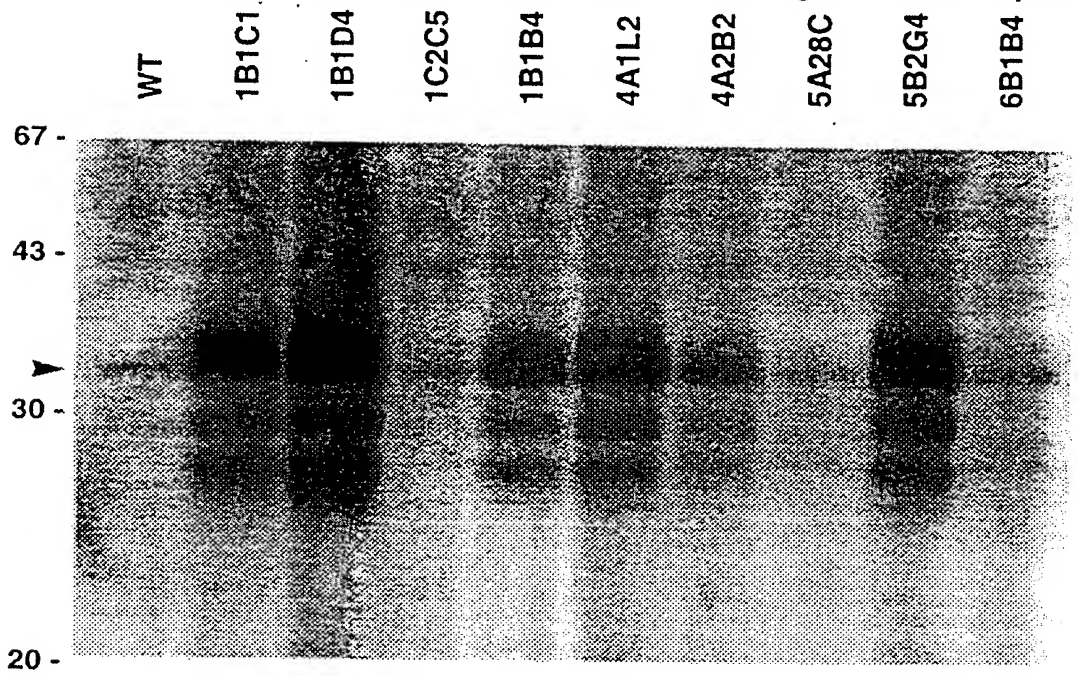


FIGURE 5

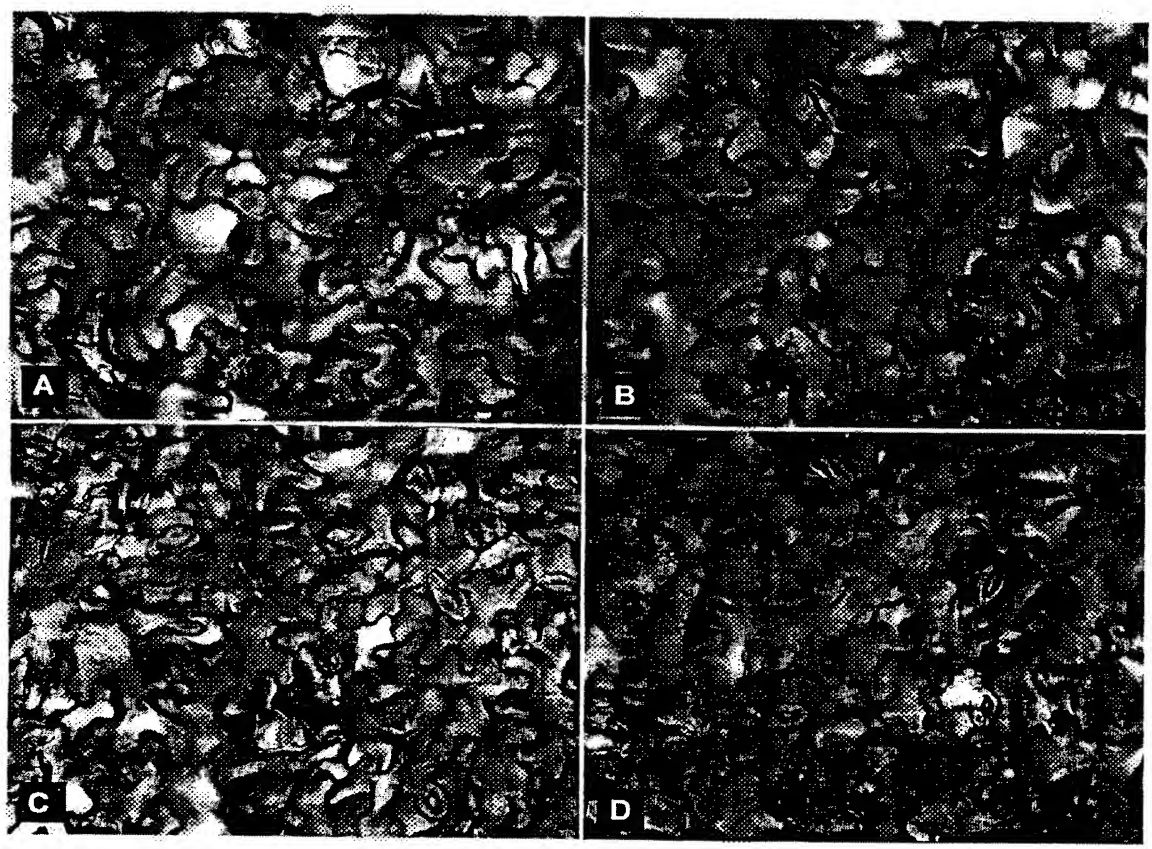


FIGURE 6

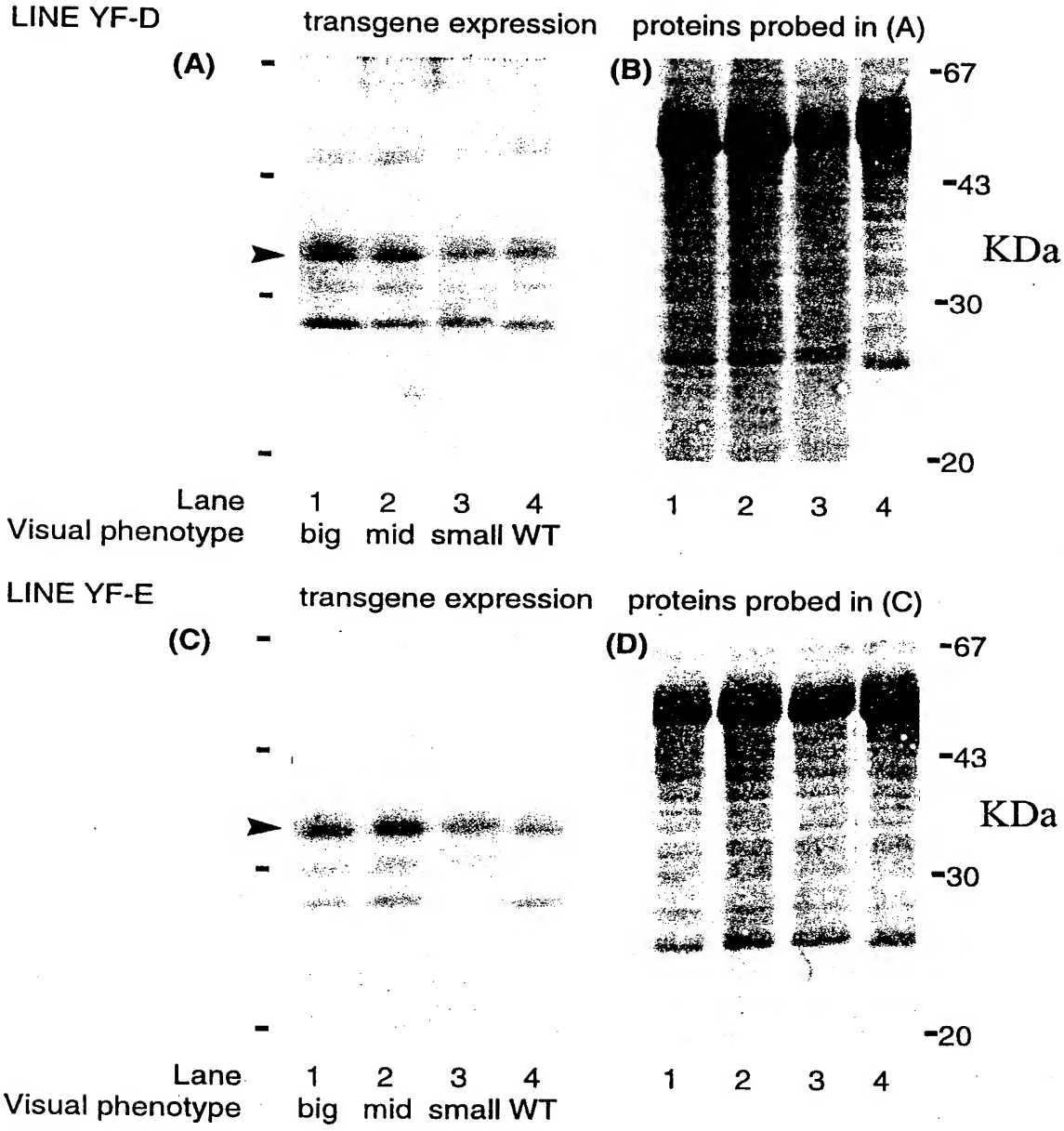

LINE YF-D
transgene expression
proteins probed in (A)
(A)
(B)
-67
-43
KDa
-30
-20
Lane 1 2 3 4
Visual phenotype big mid small WT
1 2 3 4
LINE YF-E
transgene expression
proteins probed in (C)
(C)
(D)
-67
-43
KDa
-30
-20
Lane 1 2 3 4
Visual phenotype big mid small WT
1 2 3 4

FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No. PCT/AU00/00135

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/54, A01H 1/00, C12N 9/12, C12N 15/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
see electronic databases

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
see elcetronic databases

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOTECHABS, CHEMABS, WPAT
keywords: cdc2 cdc25 plant tobacco arabidopsis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 92/09685 A (AUSTRALIAN NATIONAL UNIVERSITY) 11 June 1992	
X	whole of document	1-8, 12-19, 63-73, 75-76
Y	whole of document	74
	Hirayama T, *et al*, "Identificaiton of two cell-cycle controlling *cdc2* gene homologs of *Arabidopsis thaliana*" GENE 105 (1991) pages 159-165	
Y	whole of document	74
	WO 99/54489 A (CROPDESIGN) 28 October 1999	
P, X	whole of document	1-19, 28-29, 55-56, 65-76

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2000	3 APR 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **GARETH COOK** Telephone No : (02) 6283 2541

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00135

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 99/66055 A (CROPDESIGN) 23 December 1999 whole of document	1-14, 17, 28, 65-70

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/00135

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	92/09685	AU	90462/91	CA	2 097 286	EP	559 729
		US	5 750 862				
WO	99/54489	AU	39283/99				
WO	99/66055	AU	47725/99				

END OF ANNEX